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Composition, metabolism and mechanics of the midcarpal joint in thoroughbred horses : relationships to racing and age

Composition, Metabolism and Mechanics of the Midcarpal Joint in Thoroughbred Horses: Relationships to Racing and Age

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ABSTRACT

Disease of the midcarpal joint is common in the racing Thoroughbred (TB). Disease includes cartilage fibrillation, subchondral bone sclerosis and tearing of the medial palmar intercarpal ligament (MPICL). Exercise is one factor that greatly influences skeletal architecture, therefore, an understanding of the adaptation of the tissues to exercise may allow intervention to reduce the incidence of carpal joint disease in the racing TB. This study aimed to ascertain the mineral composition and the extracellular matrix (ECM) metabolism of the cortical and trabecular regions of radial (Cr) and third (C3) carpal bones, and the biomechanics and ECM metabolism of the dorsolateral (DL) and dorsomedial (DM) branches of the MPICL from clinically normal horses which had either been raced and race-trained, or had not raced, up to the time of euthanasia. The level of macroscopic articular cartilage erosion was also assessed. In addition, any relationships with age and the various parameters quantified were investigated.

Racing was found to result in higher bone density, and total calcium and inorganic phosphate deposition, in all regions of the Cr and C3, an adaptation to strengthen the bone. In addition, there was elevated bone collagen remodelling in the racehorses that appeared to result in a modified collagenous matrix, particularly within the trabecular regions of the bone. Failure of the 'denser' cortical bone may therefore result from its support by a metabolically more active, and therefore less stable, trabecular bone.

Relationships between articular cartilage degradation and subchondral bone changes (i.e. increased bone density) were evident, although these were most prominent within the non-racehorses. The lack of interaction of bone changes with cartilage pathology in the racehorses may be a consequence of the high-intensity exercise in an attempt to protect the bone, which in the short term is not significantly contributing to cartilage degradation.

Racing and race-training also resulted in an increased prevalence of tearing of the DL branch of the MPICL, although this did not appear to be mediated or contributing to an increased ECM metabolism, suggesting tearing is due to single traumatic event. In addition, tearing did not appear to contribute to pathology and hence gross cartilage degradation. Tearing is therefore of little clinical significance in the short term, and is probably an independent finding rather than a cause of lameness, unless the effects influence pathology in the long term.

In addition, age was found to correlate with many of the markers of collagen metabolism and to a lesser extent the inorganic properties of the Cr and C3, reflecting a reduction in the level of modelling and remodelling with maturation and growth. Interestingly, few relationships with age and the biomechanical or biochemical properties of the DL and DM branches of the MPICL were documented, which may possibly suggest that tearing or rupture of the MPICL is not a cause of degeneration with age.

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Finally, I wish to dedicate this thesis to the memory of my father and sister, whom I know would have been proud of what I have achieved.

AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Any views in the dissertation are those of the author and in no way represent those of the University of Bristol.

The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

SIGNED:.....H. Tidbury.....

DATE:.....20th July 2004.....

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CHAPTER ONE

Introduction

1.1 GENERAL INTRODUCTION

The athletic horse of today has evolved from a marsh-dwelling animal possessed of four, or even five digits per foot, to a single-digited animal. The evolutionary adaptations of the horse have enabled this prey animal to run at high speeds with maximum efficiency. A fit horse is able to maintain an average speed of 45 mph over a distance of half a mile comparable to that of only 17 mph of a human athlete (Ellis 2002). However, the athletic horse undergoes extensive and intensive training regimes, which result in repetitive loads being placed on the tissues of the joints, and each year many racehorses are lost from training and racing due to musculoskeletal injury (Hodgson and Reuben 1994). Research for all breeds of racehorses found that 84.6% of injuries were musculoskeletal in nature, about nine tenths of those injuries were in forelimbs and 90% involved the area of the limb extending from the carpus to the pastern (Johnson, Stover et al. 1994). In addition, 67.6% of lost training days have been shown to be due to lameness, with 14% of these involving the carpus (Rossdale, Hopes et al. 1985).

Disease of the midcarpal joint is common in the racing Thoroughbred (TB) and is a major cause of wastage to the racehorse industry (Rossdale, Hopes et al. 1985). Disease ultimately affects the whole of the synovial joint, including cartilage fibrillation and subchondral bone sclerosis. More recently, tearing of the medial palmar intercarpal ligament (MPICL) has also been documented (McIlwraith 1992). Emphasis on carpal joint disorders of the horse, such as, osteoarthritis (OA) and osteochondral fragmentation (OF), has previously been directed towards intra-articular events with less attention to the role of bone. Studies have shown that bone plays an important part in the pathogenesis of these diseases. Young *et al.*, (1991) have shown a relationship between the mechanical properties and morphology of equine subchondral bone and osteochondral disease in the third carpal bone (Young, Richardson et al. 1991). In addition, Norrdin *et al*, (1998, 1999) presented histological evidence for subchondral

bone failure and remodelling in an equine model of overload arthrosis (Norrdin, Kawcak et al. 1998; Norrdin, Kawcak et al. 1999), and Firth *et al.*, (1999) found that modelling of both the radial and third carpal bones occurred in young TB horses undergoing a treadmill training programme, specifically at those sites subjected to the highest loads and most commonly affected by articular cartilage pathology (Firth, Delahunt et al. 1999).

Bone, unlike cartilage and ligament, is known to respond rapidly to changes in the mechanical environment ensuring that bone mass and architecture are appropriate for the loads it is required to withstand during exercise (Rubin and Lanyon 1984). Physical exercise is one factor that greatly influences skeletal architecture and hence an understanding of the adaptation of bone to exercise may have particular relevance in reducing the incidence of joint disease in the carpus of TB racehorses.

Most of the research to date on the effect of high-intensity exercise, similar to that experienced by the racing TB, has concentrated on obtaining data on the mechanics, density and morphometry of the connective tissues (Young, O'Brein et al. 1988; Young, Richardson et al. 1991; Firth, Delahunt et al. 1999; Firth, Goodship et al. 1999; Firth, van Weeren et al. 1999; Murray, Vedi et al. 2001), primarily on treadmill exercised horses, but few have studied the extracellular matrix (ECM) metabolism of these tissues, specifically in TB horses in race-training and racing.

The overall aims of this thesis are to ascertain the composition and ECM metabolism of the radial and third carpal bones, and the biomechanics and ECM metabolism of the MPICL, so as to determine the effects of racing and race-training on these tissues within the midcarpal joint. The level of macroscopic articular cartilage erosion will also be assessed, which together will provide information on the response of the major tissues of the midcarpal joint to racing and race-training.

1.2 THE MIDCARPAL JOINT

1.2.1 Anatomy of the Midcarpal Joint

The midcarpus is composed of six axial weight bearing carpal bones (refer to Figure 1.1), although it is the radial carpal bone (Cr), the intermediate carpal bone (CI) and the third carpal bone (C3) which bear the most load during motion and that are most frequently injured. The ulnar (CU), fourth (C4) and second (C2) carpal bones are necessary for the normal function of the carpus but support smaller loads. A seventh bone, the accessory carpal bone, acts as a palmar pivot to increase the leverage of the flexor muscles' attachment to the proximal metacarpus (Bramlage, Schneider et al. 1988).

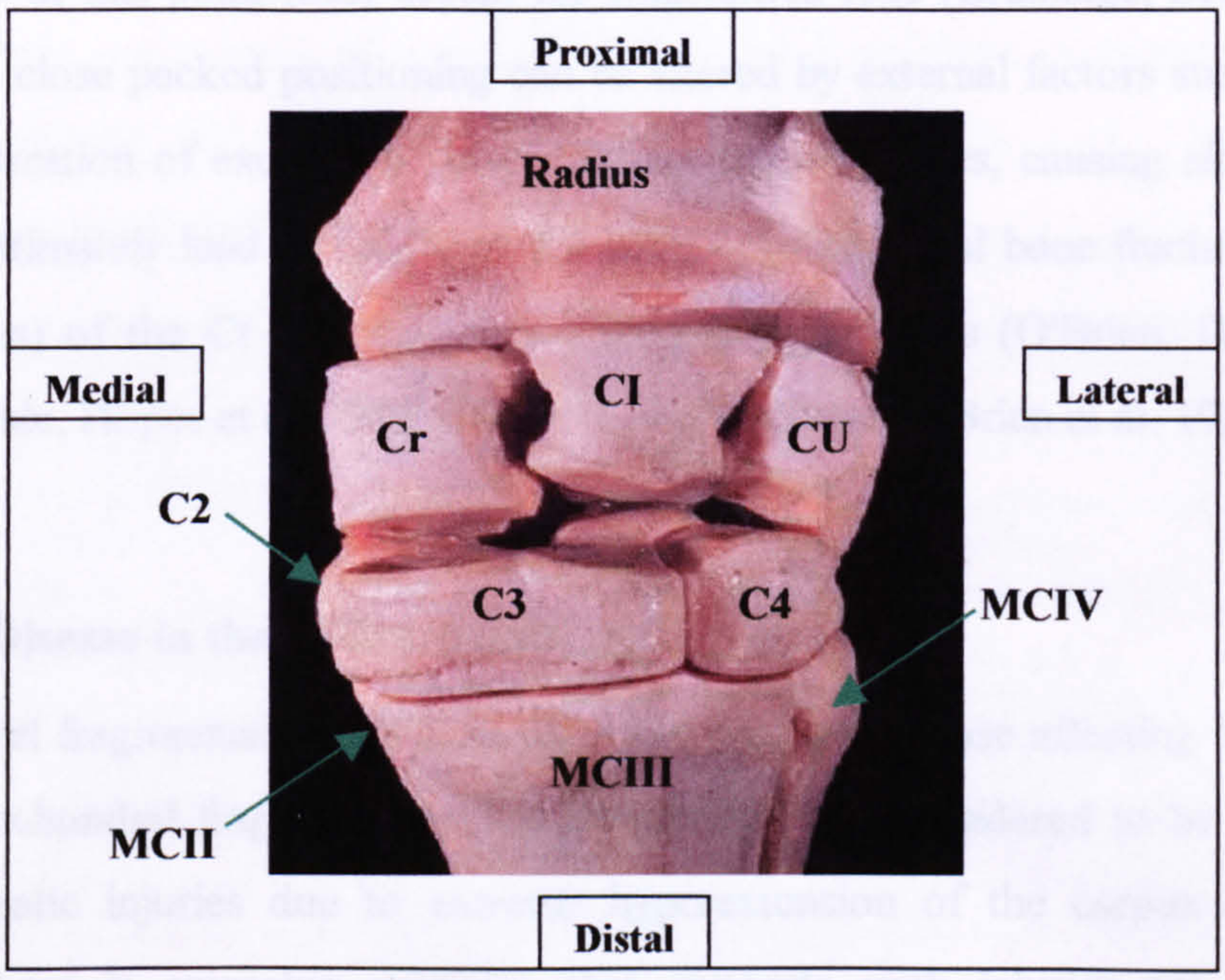


Figure 1.1: Craniocaudal (anterior) view of the equine carpus. The CU (ulnar carpal bone), CI (intermediate carpal bone), Cr (radial carpal bone), C2 (second carpal bone), C3 (third carpal bone) and the C4 (fourth carpal bone) are collectively termed the midcarpal bones. The radius, MCII (second metacarpal bone), MCIII (third metacarpal bone) and the MCIV (fourth metacarpal bone) are the additional bones of the carpus.
Source: http://www.ovcnet.uoguelph.ca/ClinStudies/Courses/OVC/93-445/lame.1/forelimb.htm
Accessed: 20th June 2004.

The carpal joint is a ginglymus, or hinge joint, in that it moves in only one direction, that of flexion and extension, without lateral or rotary movements (Smythe 1993). However, simply defining the carpus as a hinge joint does not facilitate an understanding of the biomechanical uniqueness of this joint. As Zschokke wrote back in 1892, "*the carpus of*

the horse could not be thought as a simple hinge joint because it also acts as a stress absorber" (Zschokke 1892), particularly the radial and third carpal bones.

During motion, the weight bearing stress that is transmitted from the radius into the Cr cannot be attenuated as it passes across the intercarpal joint into the distal row of carpal bones. The radial facet of the C3, unlike the other carpal articulations, is concave and receives the entire load transmitted through the Cr without the ability to dissipate any stress to soft tissue structures, such as the intercarpal ligaments (Bramlage, Schneider et al. 1988). When the carpus is fully extended the close packed weight bearing position of the carpal bones is reached, at this stage the Cr sits firmly within the radial facet of the C3. The C3 at this point must accept the transmitted load (Bramlage, Schneider et al. 1988). This close packed positioning can be altered by external factors such as fatigue, speed and duration of exercise or inappropriate track surfaces, causing altered loading which can ultimately lead to failure of the bone. Subchondral bone fractures (slab and chip fractures) of the Cr and C3 are common in racing TBs (O'Brien, DeHaan et al. 1985; Rossdale, Hopes et al. 1985; Palmer 1986; De Haan, O'Brien et al. 1987).

1.2.2 Joint Disease in the Midcarpal Joint

Osteochondral fragmentation (OF), or chip fracture, is a disease affecting the midcarpal joint. Osteochondral fragmentation has frequently been considered to be the result of acute, traumatic injuries due to extreme hyperextension of the carpus for example. However, there is emerging evidence that suggests such injuries are a secondary complication affecting joint margins previously altered by other pathological processes such as subchondral bone sclerosis, and articular cartilage erosion (Pool and Meagher 1990). These, along with osteophyte formation, are common characteristics of OA, hence OF and OA are closely linked.

It has been proposed that chip fractures of the joint margins in the carpus often arise from the following pathogenesis: Initially, subchondral bone sclerosis is induced by repetitive trauma of training and racing which, with continued exercise, leads to damage of the articular cartilage lying upon less compliant subchondral bone. Eventually, the sclerotic bone becomes ischaemic and necrotic. Osteoclasts emerging from vessels in the

adjacent viable bone invade the borders of the area of dead subchondral bone in an attempt to remodel the bone. The granulation tissue invading the bed of the chip fracture establishes a fibrous bridge between the fracture fragment and the adjacent viable bone. Continued trauma of racing destabilises the fragment and failure results (Pool and Meagher 1990).

1.2.3 Composition of the Tissues of the Midcarpal Joint

Bone:

Bone is an extremely important tissue of the body, fundamentally providing support, movement and protection. It is composed of an inorganic component of mineral salts and an organic component of predominantly type I collagen, proteoglycans (PGs), glycosaminoglycans (GAGs), water and cells (refer to Table 1.1). Blood vessels, lymphatic vessels and nerves are also highly abundant in bone.

Composition of Bone	
Mineral ~70% dry weight	Hydroxyapatite
Organic Matrix ~30% dry weight	Collagen ~90% of the matrix Proteoglycans and glycoproteins Lipids and carbohydrates
Cells	Osteoblasts (synthesise bone matrix) Lining cells Osteoclasts (resorb bone) Osteocytes
Water	~10-15%

Table 1.1: The composition of bone. Adapted from Christian *et al.*, (1998).

The organic matrix of bone constitutes about 30% of the dry weight of bone. It consists of 90% collagen, which is thus by far the most abundant bone protein. This collagen is almost exclusively type I collagen, with small amounts of type II, V, VI and XII. The inorganic mineral represents about 70% of the total dry weight of bone. Specifically, the mineral structure is like that of hydroxyapatite, which has the composition $Ca_{10}(PO_4)_6(OH)_2$ (Christiansen, Hassager et al. 1998; Boskey, Wright et al. 1999). The inorganic phase imparts a hardness, rigidity and compressive strength to the bone, whereas the organic phase provides flexibility, resiliency and tensile strength (Liu, Yang et al. 1995).

The bones of the carpus are short bones that are composed of subchondral bone and trabecular bone. The subchondral bone can be further classified into the cortical bone plate (or subchondral bone plate) and trabecular bone (Imhof, Sulzbacher et al. 2000; Kawcak, McIlwraith et al. 2001). The cortical bone plate is defined within this thesis as the bone layer separating the articular cartilage (specifically the deeper calcified cartilage) from the subchondral trabecular bone.

Articular Cartilage:

Articular cartilage is a tough but flexible connective tissue, providing resilient rigidity to subchondral bone and is essential for friction free movement, absorbing shock and maintaining normal joint environment. Articular cartilage is avascular and devoid of nerve fibres, so it receives most of its nutrients and limited blood supply from the underlying subchondral bone (Imhof, Sulzbacher et al. 2000). Its ground substance contains large amounts of PGs (40% dry weight), glycoproteins (10% dry weight) comprising fibronectin and chondronectin (adhesion proteins), cartilage oligomeric matrix protein (COMP) and growth factors, and is heavily invested with firmly bound collagen fibres (50% dry weight). Type II collagen is the predominant collagen type within cartilage (85 to 90% of the total), although types VI, IX, XI, XII and XIV are also present. The cartilage matrix also has a high water content, comprising of up to 70% water. The PGs and GAGs, due to their ability to bind large volumes of water relative to their molecular weight, control the water content within the cartilage and thus affect the viscoelastic properties of the tissue (Viidik, Danielson et al. 1982). Chondrocytes are the single cell type found in cartilage and make up 1% to 12% by volume (Marieb 1995; Todhunter 1996).

Ligament:

Ligaments are short bands of dense, white, homogenous, tough connective tissue that link bones together or hold organs in place. They are predominantly composed of well orientated dense collagen fibres, this being almost exclusively type I collagen, which makes up approximately 80% of the dry weight and provide the major resistance to mechanical loading. Collagen types II, V and VI are also present, but in much smaller quantities. The collagen fibres are densely packed, running in the same direction, parallel

to the direction of pull. This results in a flexible tissue with great resistance to tension (Marieb 1995).

The remaining 20% of ligament tissue consists of elastin fibres enmeshed in a mucopolysaccharide ground substance containing PGs, other minor ECM proteins, such as COMP (a noncollagenous ECM protein) and cells. These cells are predominantly fibroblasts, which are commonly known as ligamentocytes in ligaments (Frank, Amiel et al. 1985; Vasseur, Pool et al. 1985). Elastin probably accounts in part, for the tensile resistance in ligament tissue and some of its elastic recoverability. In its fibrillar form, elastin is arranged in a complex coil, which is stabilised by lysine-derived cross-links. When stressed, elastin stretches out to a more ordered configuration and reverts to the globular formation when the stress is removed (Viidik, Danielson et al. 1982).

Limited information exists on the ultrastructure of the MPICL. Whitton *et al.*, (1999) have described its histopathological characteristics. In this study, the collagen branches within the ligaments were found to be generally disorganised and with poor alignment. The ligaments were highly cellular, with a moderate number of small blood vessels. The predominant cell type was the fibroblast, which had a variety of cell shapes (Whitton and Rose 1999).

1.3 THE EFFECTS OF EXERCISE ON BONE

It is recognised that a degree of exercise can have beneficial effects on bone, with studies reporting increases in bone density, mass and strength in the horse (Jeffcott 1988; McCarthy and Jeffcott 1988; Buckingham, McCarthy et al. 1992; Reilly, Currey et al. 1997; Firth, Goodship et al. 1999), in humans, and in experimental animals (Dalen and Olsson 1974; Pirnay, Bodeux et al. 1987; Raab, Smith et al. 1990; Shibata, Ohsawa et al. 2003; Nurmi-Lawton, Baxter-Jones et al. 2004). However, these beneficial effects appear to be dependent upon the age of the animal, and the intensity, speed and duration of exercise (McCarthy and Jeffcott 1991; Bennell, Malcolm et al. 1997; Boston and Nunamaker 2000; Robling, Hinant et al. 2002).

Bennell *et al.*, (1997) have documented in human athletes that the response of bone to mechanical loading depends upon the bone site and mode of exercise. Changes in bone mineral density (BMD) were found to be independent of exercise status in the lower and upper limb but not in the lumbar spine where, at this site, power athletes (sprinters, multi-event athletes) gained significantly more bone density over a twelve month period compared to endurance athletes (middle-distance runners) and non-athlete controls (Bennell, Malcolm et al. 1997). Additionally, Raab *et al.*, (1990) demonstrated that bone fat-free weight (FFW) and bone strength increased significantly with training in rats. Both young (2.5 months) and old (25 months) trained rats had significantly greater femur and humerus FFW and greater yield and ultimate strength, measured using a three-point bending test, compared to young and old untrained rats (Raab, Smith et al. 1990). In a study by Boston *et al.*, (2000) to determine which components of a training program in two year old TB racehorses influenced their susceptibility to fatigue injury of the third metacarpal bone (MCIII) (i.e. 'bucked shins'), it was shown that those horses subjected to regular short-distance 'breezing' (moderate speed) had less incidence of fatigue injury during the one year of monitoring, than those horses which had been exercised by long-distance galloping (high speed). The conclusion was that speed and exercise intensity increases the susceptibility to fatigue injury in the MCIII (Boston and Nunamaker 2000).

High-intensity exercise may therefore have adverse effects on bone, which may ultimately be detrimental to the integrity of the bone (Bourrin, Genty et al. 1994; Wohl, Boyd et al. 2000). In treadmill exercised rats, strenuous exercise resulted in a significant decrease in femoral neck material properties. The altered bone structure and reduced material properties resulted in a significant reduction of energy at the proportional limit in the exercised rat femoral neck (Wohl, Boyd et al. 2000). In the horse, new bone formation, quantified using Computed Tomography (CT) and radiography, in the right MCIII of high-speed treadmill exercised two year old TBs was found to be increased in the highly loaded aspects of the medial and lateral condyles of the MCIII compared to non-exercised controls, which the authors proposed may lead to concentration of strain at the condylar grooves, a common site of fracture, and incipient cracking through calcified cartilage and into the subchondral bone (Riggs and Boyde 1999). This local response variation to high-intensity treadmill exercise has also been documented in the Cr, C3 and

CI, with BMD being markedly greater in the treadmill exercise horses compared to controls, and at those sites most commonly affected with pathology (Firth, Delahunt et al. 1999; Murray, Vedi et al. 2001). However, a degree of increased bone density may be a normal adaptive modelling and remodelling response to increased mechanical demand in horses with exercise i.e. exercised induced sclerosis (Butler, Colles et al. 1993), possibly in an attempt to increase bone strength.

High-intensity treadmill exercise has also been shown to result in an increase in serum and urinary biomarkers of bone turnover in the horse (Price, Jackson et al. 1995; Hiney, Potter et al. 2000; Vervuert, Coenen et al. 2002; Billinghurst, Brama et al. 2003). These biomarkers include, osteocalcin, a marker of bone formation, and the pyridinoline cross-linked telopeptide domain of type I collagen (ICTP), a marker of bone resorption. In most studies these biomarkers were found to generally decline as training continued, possibly reflecting the acute responsiveness of bone to increased exercise.

Exercise has also been documented to result in a modified collagenous matrix in foals. Collagen is the main ECM protein in bone, which together with mineral, governs the biomechanical properties and functional integrity of the tissue (Burstein, Zika et al. 1975; Einhorn 1992). The organic matrix of subchondral bone was quantified in two sites of the metacarpophalangeal (MCP) joint of foals aged five months that had been subjected to a daily exercise programme consisting of an increasing number of gallop sprints. An altered collagen composition, primarily, increased mature collagen cross-link and hydroxylysine content, was found at the site most loaded during high speed exercise compared to non-exercised foals (Brama, Bank et al. 2001; Brama, TeKoppele et al. 2002). Collagen cross-links and the level of hydroxylysine alter the mechanical strength of the collagen network and hence play a vital role in the biomechanical integrity of the bone (Knott, Whitehead et al. 1995; Oxlund, Barckman et al. 1995). Any alteration in the collagenous matrix of bone may therefore impair the biomechanical properties of the joint tissue. This in turn, may lead to failure of the bone, since it is unable to cope with the compressive, shear and tensile forces to which the joint is continuously subjected to during high-intensity exercise.

1.3.1 The Response of Bone to Exercise with Age

Exercise at a young age is one factor that may affect skeletal development (Smith, Birch et al. 1999) and be involved in the occurrence of specific lesions such as OF, OA and osteochondrosis (OC) in the horse (Jeffcott 1991; Jeffcott and Henson 1998). A comprehensive study evaluating the influence of exercise on the development of the musculoskeletal system of foals up to the age of eleven months by van Weeren *et al.*, (van Weeren and Barneveld 1999) found that at five months of age BMD in the C3, distal radius (Firth, van Weeren et al. 1999), third metatarsal bone (MTIII) (Barneveld and van Weeren 1999), MCIII, and the proximal sesamoid bone (Cornelissen, van Weeren et al. 1999) was significantly higher in those foals that had either been subjected to daily sprint training or kept at pasture (exercised) compared to box-confined controls (non-exercised). The effect of exercise was most evident in the trabecular bone of the proximal sesamoid (Cornelissen, van Weeren et al. 1999) but this is not unexpected since the turnover rate of trabecular bone is about eight times higher than that of cortical bone (Scotti and Jeffcott 1988). The data from these studies suggest that the lack of exercise at an early age results in a retardation of normal development of bone. However, when exercise was introduced to the box-confined foals at five months until eleven months of age the difference observed between the exercised and non-exercised group at age five months was no longer present in the horses at eleven months, suggesting that this delay in normal development is compensated for when the restriction of exercise is lifted. Interestingly, BMD in the sprint-trained group at eleven months was found to decrease below the levels found in the pasture group, particularly in the trabecular bone of the proximal sesamoid bone (Cornelissen, van Weeren et al. 1999), suggesting that the exercise that was demanded during the first five months of life may have resulted in an (over)stimulation of the osteoblasts resulting in less active mineral deposition in the longer term. Thus it can be concluded that high-intensity exercise at an early age may have long-lasting negative effects on the quality of the bone. Furthermore, the results from these studies suggest that sprint-training exercise appears to have a larger effect on BMD than regular sub-maximal exercise (pasture exercise), leading to the conclusion that factors such as speed, exercise intensity, impact and strain rate are more important than duration of exercise.

McCarthy *et* al., (1991) also found bone quality and quantity to be altered in the mid-metacarpus of yearling TB horses subjected to treadmill exercise compared to controls. Microradiography and histomorphometry demonstrated reduced bone porosity and increased dorsal thickness in the exercised foals; ultrasound speed and bone mineral content (BMC) were also increased. Such differences with exercise were not observed in two year old or adult horses. These results suggest that high-intensity exercise in young horses may cause a reduction in intracortical remodelling activity and alteration in the cross-sectional morphology of the mid-metacarpus by modelling (McCarthy and Jeffcott 1991).

To date, no information exists on the effects of high-intensity exercise on bone density, morphometry and collagen metabolism of maturing and mature horses, specifically those horses in racing and race-training.

1.3.2 The Response of Bone to Loading and Exercise

In order to gain an understanding of exercise-related articular injuries, it is important to develop an appreciation of the balance between joint adaptation and injury during high-intensity exercise. Within the following section, the response of bone to changes in its mechanical environment and how this could ultimately lead to failure will be reviewed.

1.3.2.1. Basic Bone Mechanics

During high-intensity exercise the loads that are absorbed by the bones in the distal limb of the horse are extensive, often withstanding strain rates which are very close to the limit of their ultimate strength (Evans, Behiri et al. 1992). When this load is applied to the bone, it deforms and within certain limits this deformation is within the elastic limit of the bone and the bone will return to its original shape, unaltered, once the load has been removed. However, if the degree of deformation rises beyond a threshold limit, irreversible change may be caused to the bone (Lanyon 1992a; Lanyon 1992c). A single extreme load could possibly deform the bone beyond its ultimate limit resulting in complete and sudden failure. Alternatively, while the extent of damage from a lesser load may be insufficient to cause failure, repetitive loading, as occurs to the midcarpal

joint during racing, may result in cumulative (fatigue) damage, which may ultimately lead to catastrophic failure.

The number of load cycles to failure (or fatigue-life) of a bone, is logarithmically related to the extent of deformation (strain) with each load cycle (refer to Figure 1.2). The degree of deformation is a function of the magnitude of the load and stiffness of the bone. The stiffness of the bone being ultimately determined by the material properties from which it is formed (Riggs 2002).

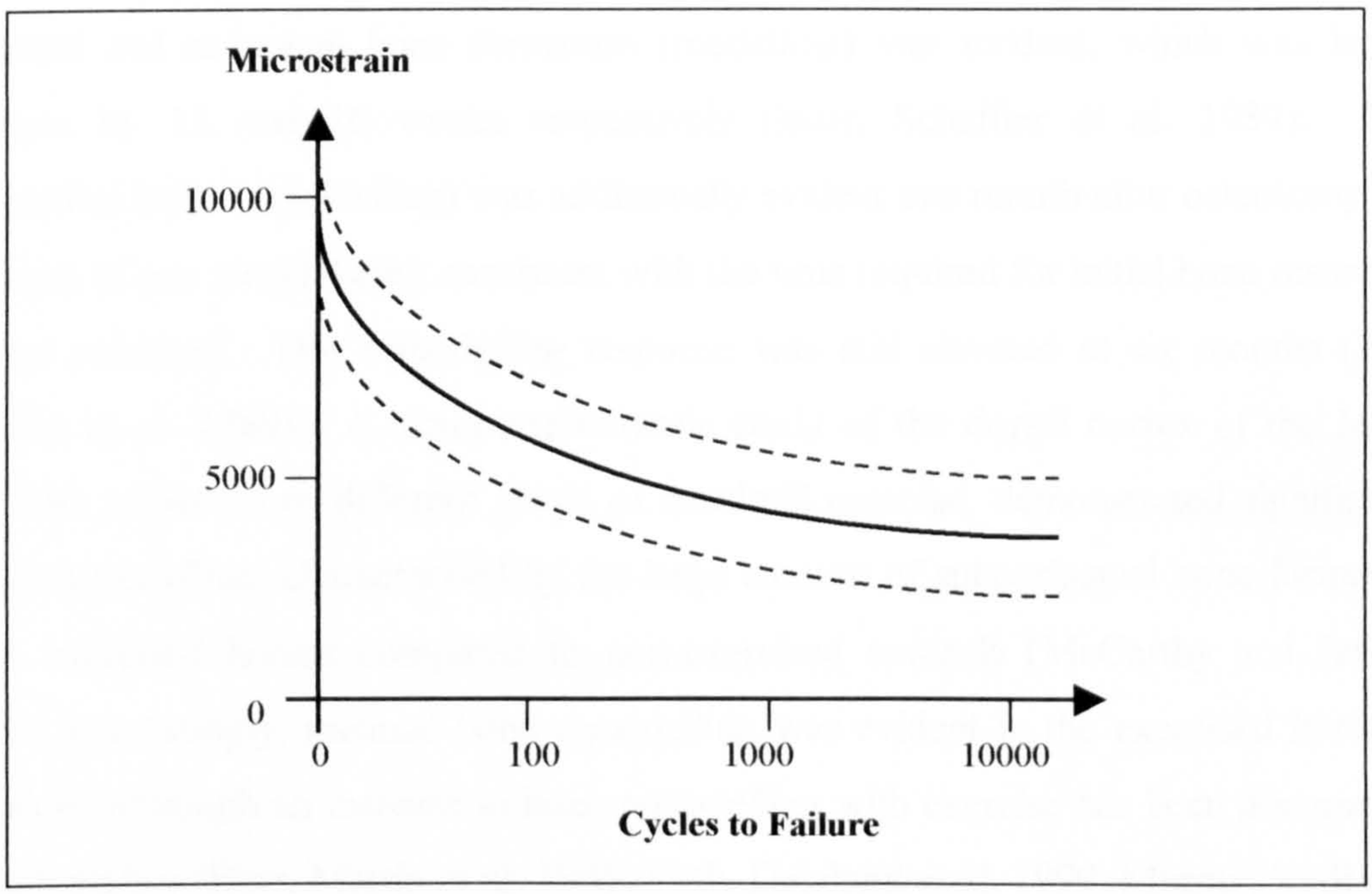


Figure 1.2: The number of cycles to failure against strain per cycle for bone from the Thoroughbred third metacarpal bone (Nunamaker, Butterweck et al. 1987).

1.3.2.2. Bone Modelling and Remodelling

The overall formation of bone is genetically predetermined, however, its mass, three dimensional structure and microstructure are capable of changing in response to alterations in its mechanical environment (Lanyon 1987; Riggs, Vaughan et al. 1993). This is achieved by the process of bone modelling and remodelling (Frost 1991; Burr 1993; Kawcak, Norrdin et al. 1998). Modelling refers to the formation (synthesis) or

resorption (degradation) of bone on an existing surface, thus changing bone shape to withstand the loads placed on it. Remodelling refers to the removal of bone from one location and its replacement at the same location in response to loading or microdamage (Frost 1991). Bone remodelling involves a continuous balance and communication between cell populations and their associated activation factors and cofactors, the process of which will be reviewed in section 1.7.2.

It is not known how long it takes in the horse for a bone to complete adaptive modelling/ remodelling following a significant change in mechanical loading. When the mechanical environment of the canine radius was significantly altered by ulnar osteotomy, reactive periosteal and endosteal bone formation (modelling) was evident, which was largely complete by 12 and 16 weeks respectively (Burr, Schaffler et al. 1989). New intracortical bone (remodelling) was additionally evident one month after osteotomy, this lag-phase of one month being consistent with the time required for initial bone resorption to have occurred. This remodelling response was still elevated at six months (Burr, Schaffler et al. 1989). A histomorphometric study of the dorsal cortex of the MCIII from TBs subjected to different levels of treadmill exercise, demonstrated significantly increased modelling, characterised by the large amount of subperiosteal bone formation, in the exercised horses compared to non-exercised controls (McCarthy and Jeffcott 1992). Interestingly, minimal bone remodelling was evident in the exercised horses in this study, although an increase in bone remodelling with exercise has been documented in other studies (Burr, Martin et al. 1985; Firth, Delahunt et al. 1999; Murray, Vedi et al. 2001). This lack of intracortical remodelling was thought to explain the increased bone density evident within the exercised horses (McCarthy and Jeffcott 1992). Unfortunately, the time-scale of the bone modelling response was not defined but this study demonstrates that bone formation is elevated in response to increased exercise. More recently, a study by Firth *et al.*, (1999) examining the BMD in the dorsal aspect of the C3 of two year old TBs exercised on a treadmill, demonstrated that 4.5 months of exercise produced a significantly greater volumetric BMD compared to non-exercised controls, indicating that an osteoinductive response to exercise is rapid (Firth, Goodship et al. 1999).

1.3.2.3. Bone Microdamage

The mechanism by which an increase in mechanical loading induces bone change is unknown. Bone microdamage has been shown experimentally to stimulate bone remodelling (Mori and Burr 1993; Burr and Schaffler 1997). Studies using a model of fatigue damage in the anaesthetised rat have demonstrated a relationship between microdamage, osteocyte apoptosis and osteoclastic resorption (Verborgt, Lundin-Cannon et al. 2000). The sequence of events indicates that osteocytes die as a consequence of local microdamage and this, in turn, is the stimulus for bone resorption (Verborgt, Lundin-Cannon et al. 2000).

Microdamage, in the form of microcracks, has been induced both *in vivo* and *ex vivo* subsequent to cyclic loading of the bone (Martin, Stover et al. 1996), with studies demonstrating an increase in crack density, but not length, with continued loading (Martin, Stover et al. 1996). The formation of microcracks has been suggested to increase the toughness of bone and hence its resistance to failure. This is because the energy absorbed in the creation of many small cracks is diverted from extension of a single large crack (Reilly, Currey et al. 1997). Reilly *et al.*, (1997) found that the amount of microcracking produced during mechanical testing of the MCIII of treadmill exercised TBs positively correlated with strength (Reilly, Currey et al. 1997). However, with continued loading, an over accumulation of microcracking and increased density of the microcracks will inevitably lead to failure of the bone (Riggs 2002). Furthermore, microcracks have been associated with a loss of bone stiffness due to the increased deformation of the bone in response to loading, thus impairing the mechanical properties of the bone (Burr, Turner et al. 1998).

It is the process therefore between bone resorption and formation (remodelling) that allows bone to respond to the increased demands during exercise, possibly in an attempt to increase strength. However, if exercise continues, microdamage formation may exceed remodelling repair, and eventual failure of the articulating joint may result (Burr 1993).

1.4 THE ROLE OF BONE AND ARTICULAR CARTILAGE IN JOINT DISEASE

1.4.1 The Initiating Component in Joint Disease

Articular cartilage, being composed of chondrocytes surrounded by an extracelluar matrix of collagen, PGs and water, is supported by subchondral bone. Its molecular composition enables it to absorb some of the load applied to the bone during loading and it is the balance between the tension in the collagen network and the osmotic swelling pressure of the PGs that provides the biomechanical characteristics of the articular cartilage essential for normal joint function (Palmer and Bertone 1996). In numerous studies, it has been shown that in exercised horses gross lesions in the articular cartilage are significantly more severe than in non-exercised controls (Brama, Tekoppele et al. 2000), and subchondral bone sclerosis is often observed underlying sites most commonly affected by cartilage pathology (Firth, Delahunt et al. 1999). The relative importance of articular cartilage and/or subchondral bone in the pathogenesis of joint diseases, such as OA and OF, in humans and the horse is subject to debate. Two theories are currently proposed. The mechanical stress on weight-bearing joints, such as the midcarpus, may contribute to an increase in microfractures in the subchondral bone plate and overlying cartilage. As the articular cartilage slowly erodes, sclerosis of the subchondral bone plate also progresses and bone stiffness increases, possibly contributing to further mechanical disturbances of the cartilage (Lajeunesse, Hilal et al. 1999). Conversely it is known that subchondral bone, rather than cartilage absorbs most of the impact under loading (Radin and Paul 1970) and increased mechanical loading of bone suppresses the resorption phase of remodelling leading to trabecular thickening or sclerosis. This sclerosis increases the stiffness of the bone and transfers the forces back to the cartilage. Those that argue that bone changes are primary in the pathogenesis of joint disease feel that this initiates degeneration in the cartilage (Radin and Paul 1970; Radin, Paul et al. 1970; Radin and Rose 1986).

Radin *et al.*, (1984) have shown experimentally in rabbits that osteoarthritic changes, i.e. cartilage erosion, could be induced within 8 weeks of impulsive loading of the tibia followed by 24 weeks of rest. When studied after 6 weeks, increases in calcified cartilage thickness and subchondral bone remodelling were evident, but there were no changes in cartilage PG properties, including size and concentration of PG and synthetic

rate of GAGs (Radin, Martin et al. 1984). Thickening of the subchondral bone has also been shown radiographically to occur prior to cartilage degeneration (Carlson, Loeser et al. 1996). Microfocal X-radiography has also shown, in a guinea pig model of spontaneous OA, that in both the lateral and medial sides of the tibia, the subchondral bone was denser at 24 weeks and 36 weeks of age, the time when very early histopathological OA signs i.e. changes in cartilage surface and acellularity are occurring, thus indicating that bone density is not simply a response to advanced cartilage damage (Anderson-MacKenzie, Quasnichka et al. 2003).

However other studies have shown that cartilaginous changes may be occurring before gross clinical signs are evident. Price *et al.*, (1999), have shown that in the cartilage of patients with anterior cruciate ligament (ACL) injury but no clinical signs of OA, there was a significant increase of type II collagen degradation, similar to that observed in the cartilage of patients with late-stage OA (Price, Till et al. 1999). In addition, changes to the cartilage composition with exercise have been shown to occur before gross signs of degeneration are evident. Arkoski *et al.*, (1993) found that a 12 month 40-km/day running exercise programme in beagle dogs resulted in a depletion of GAG concentration, predominantly at weight-bearing areas of the femoropatellar joint, without signs of cartilage degeneration (Arokoski, Kiviranta et al. 1993).

Clearly initiation of the pathogenesis of joint disease is subject to much debate, but probably involves interactions between all elements of the synovial joint (Senior 2000).

1.4.2 Bone Collagen Metabolism

Regardless of the initiating event(s) that cause joint disease, changes to the subchondral bone are a distinguishable feature in joint disease. Changes in the collagen metabolism in both the subchondral bone plate and the subchondral trabecular bone of osteoarthritic and osteoporotic human femoral heads have been recently documented (Bailey, Wotton et al. 1993; Bailey and Mansell 1997; Mansell, Tarlton et al. 1997; Mansell and Bailey 1998; Mansell and Bailey 2003). Bailey *et al.*, (1997) demonstrated in OA femoral heads that concentrations of collagen degrading enzymes, matrix metalloproteinases (MMP-2), and concentrations of carboxyterminal propeptide of type I collagen (PICP)

and alkaline phosphatase, markers of bone degradation and synthesis respectively, were elevated in both the subchondral bone plate and the trabecular bone compared to non-OA controls, with these concentrations being greatest in the subchondral plate (Bailey and Mansell 1997). In addition, it has been shown that the collagen content is increased and conversely the mineral content decreased in osteoarthritic femoral heads compared with non-OA controls, suggesting a greater proportion of osteoid (unmineralised new bone matrix) in the diseased bone (Mansell and Bailey 1998). The consequence of this hypomineralisation is a decrease in the overall strength and compressive stiffness of the bone (Mansell and Bailey 1998). These results demonstrate that subchondral bone collagen turnover is elevated in diseased tissue, which may lead to altered biomechanics of the bone and subsequently exacerbate the disease process.

Studies have also demonstrated that during the formation of collagen cross-links in osteoporotic bone (characterised by low bone mass and strength, with a consequent increase in bone fragility) there is an increase in the hydroxylation of lysine residues, which is likely to result in narrower collagen fibres. This could lead to a decrease in strength of the collagen framework and ultimately the tensile strength of the bone (Bailey, Wotton et al. 1992; Bailey, Wotton et al. 1993; Knott, Whitehead et al. 1995; Bailey and Knott 1999).

1.5 THE LIGAMENTS OF THE MIDCARPAL JOINT

1.5.1 Structure, Function and Clinical Relevance

The development of diagnostic and surgical arthroscopy has allowed for the accurate assessment of previously less well-defined anatomical structures within equine joints as well as pathological conditions of the same structures (Hurtig and Fretz 1986). During arthoscopic removal of osteochondral fractures in the midcarpal joint, tearing of the palmar intercarpal ligaments has been observed which may be of clinical significance. The medial palmar intercarpal ligament (MPICL), the lateral palmar intercarpal ligament (LPICL) and the dorsomedial intercarpal ligament (DMICL) are all ligaments of the equine midcarpal joint. Whitton *et al.*, (1997) have described their anatomy (refer to Figure 1.3a) (Whitton, McCarthy et al. 1997).

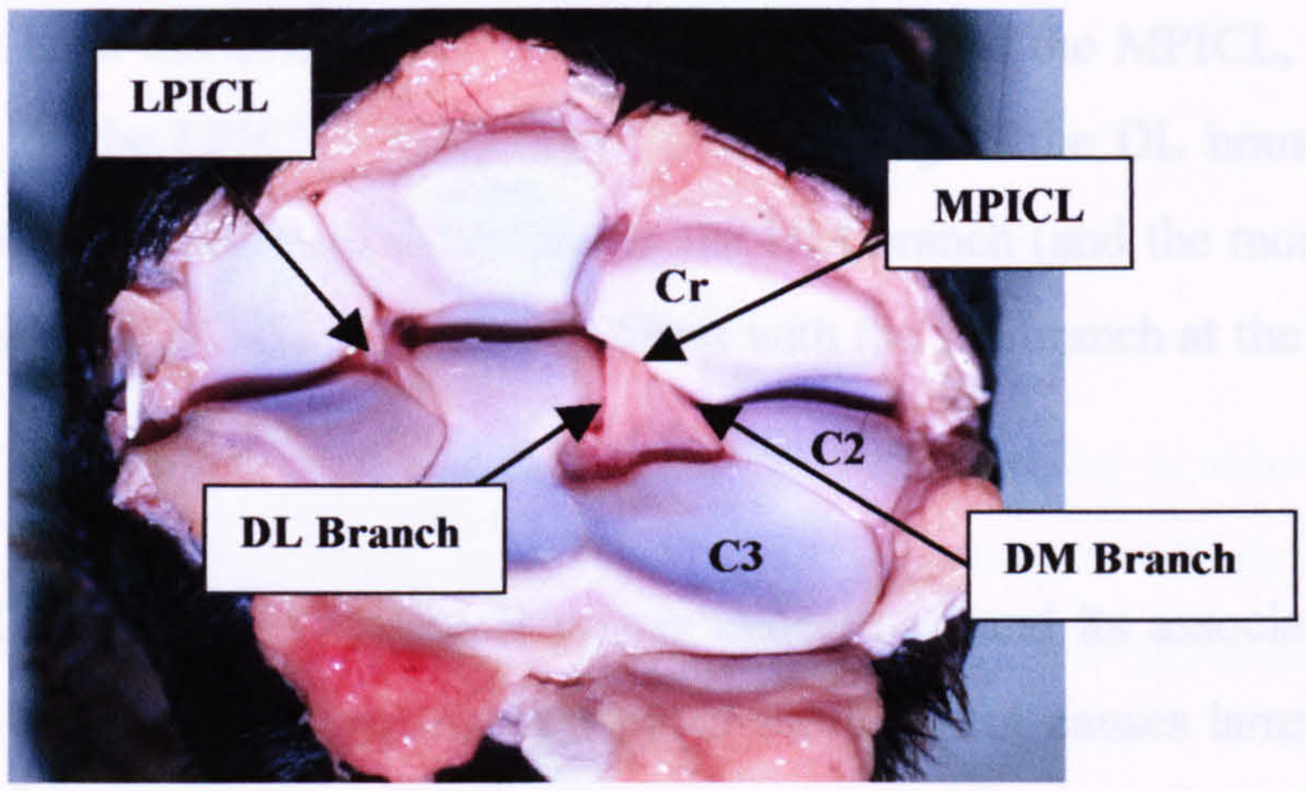
- **MPICL** - Attaches proximally to the distolateral surface of the Cr and distally to the proximal palmaromedial surface of C3 and the proximal palmarolateral aspect of C2. The MPICL is divided into four fibre branches, the dorsolateral (attaches Cr-C3 dorsally), dorsomedial (attaches Cr-C2 dorsally), palmarolateral (attaches Cr-C3 palmarly) and palmaromedial (attaches Cr-C2 palmarlly) (refer to Figure 1.3b).

- **LPICL** – Attaches proximally on the distal part of the palmaromedial surface of the CU.

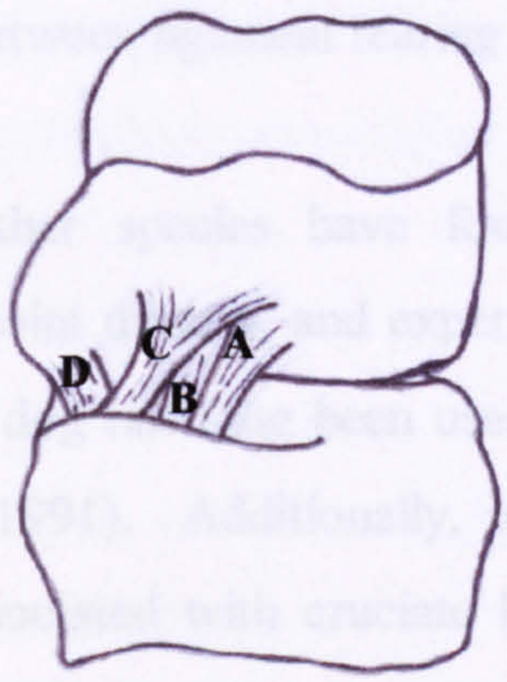
- **DMICL** – Arises from the dorsomedial surface of the Cr and courses palmarodistally to insert on the dorsomedial aspect of the C2.

It has been proposed that these intercarpal ligaments act to dissipate the axial loads applied to the carpus during high-intensity exercise and resist the lateral and medial displacement of the carpal bones, ultimately serving to protect the carpal bones (Bramlage, Schneider et al. 1988).

a) The Midcarpal Ligaments



b) The Branches of the MPICL



A= Dorsolateral branch (DL)

B= Dorsomedial branch (DM)

C= Palmarolateral branch (PL)

D= Palmaromedial branch (PM)

Figure 1.3: a) Photograph of the midcarpal joint of the horse, illustrating the Lateral Palmar Intercarpal Ligament (LPICL) and the Medial Palmar Intercarpal Ligament (MPICL) and b) a diagram illustrating the branches of the MPICL. Adapted from Whitton *et al.*, (1997).

Kannegieter *et al.*, (1990) have documented tearing and/or pathology of both the LPICL and the MPICL (Kannegieter and Burbidge 1990), whereas McIlwraith (1990) has only reported tearing and/or pathology of the MPICL (McIlwraith 1990). Since this time, many other authors have documented the incidence of tearing of the MPICL, with less evidence of tearing of the LPICL. More specifically, tearing of the DL branch of the MPICL is more frequently observed compared to the DM branch (and the more palmar branches) (McIlwraith 1992), which has shared fibres with the DL branch at the proximal end.

The cause of MPICL injury and tearing is poorly understood and its association with carpal joint disease uncertain. Complete rupture of the ligament causes lameness that can be localised to the midcarpal joint (Whitton, Kannegieter et al. 1997), but the mechanism by which damage to the ligaments causes lameness is not clear. It has been proposed that instability occurs in the joint because of ligament damage, particularly if complete rupture occurs, and that this predisposes to abnormal weight bearing and stresses on the joint, possibly leading to osteoarthritic changes in the tissues of the joint (Kannegieter and Colgan 1993). However, a high prevalence of MPICL tearing has been observed in horses with no history of joint disease (Whitton and Rose 1997), hence the relationship between ligament tearing and lameness is unclear.

Studies in other species have found that intra-articular ligament damage causes degenerative joint disease, and experimental transection of the cranial cruciate ligament (CCL) in the dog has long been used as a model for demonstrating stifle OA (Brandt, Myers et al. 1991). Additionally, in the guinea pig model of spontaneous OA, joint instability, associated with cruciate ligament alteration has been shown to lead to the early onset of OA (Anderson-MacKenzie, Billingham et al. 1999; Quasnichka 2002). In a study by Quasnichka (2002) a strain of guinea pigs that develop knee OA (Dunkin-Hartley (DH) earlier than the control (Bristol Strain 2 (BS2), were found to have more lax cruciate ligaments, which may cause relative joint instability and predispose this strain to early OA. Biochemical alterations were also documented, with the DH having increased MMP-2, immature cross-links and PICP (markers of collagen remodelling) in the cruciate ligaments, which may have caused this increased laxity (Quasnichka 2002).

This in turn may lead to a change in the stress on the bone and cartilage, resulting in altered loading on these tissues.

1.6 THE MECHANICAL PROPERTIES OF LIGAMENTS

1.6.2 The Biomechanical Properties of Ligaments

The functional or mechanical behaviour of all ligaments under loading is determined by the structural orientation, proportion and properties of the collagen and elastin fibres (Cabaud 1983). Tensile strength testing of ligaments to failure can therefore provide information on the structural and material properties of the ligament.

1.6.2.1 Tensile Behaviour

Ligaments can be pulled at a constant rate of deformation, and the force required to maintain this deformation rate is measured. The force is then plotted against the extent of ligament deformation during testing. This force or load deformation curve for a tested ligament is not linear. Several regions of the curve can be identified and related directly to morphological changes of the ligament during testing as summarised below and in Figure 1.4. The different mechanical terms used in the following are defined in Appendix Six.

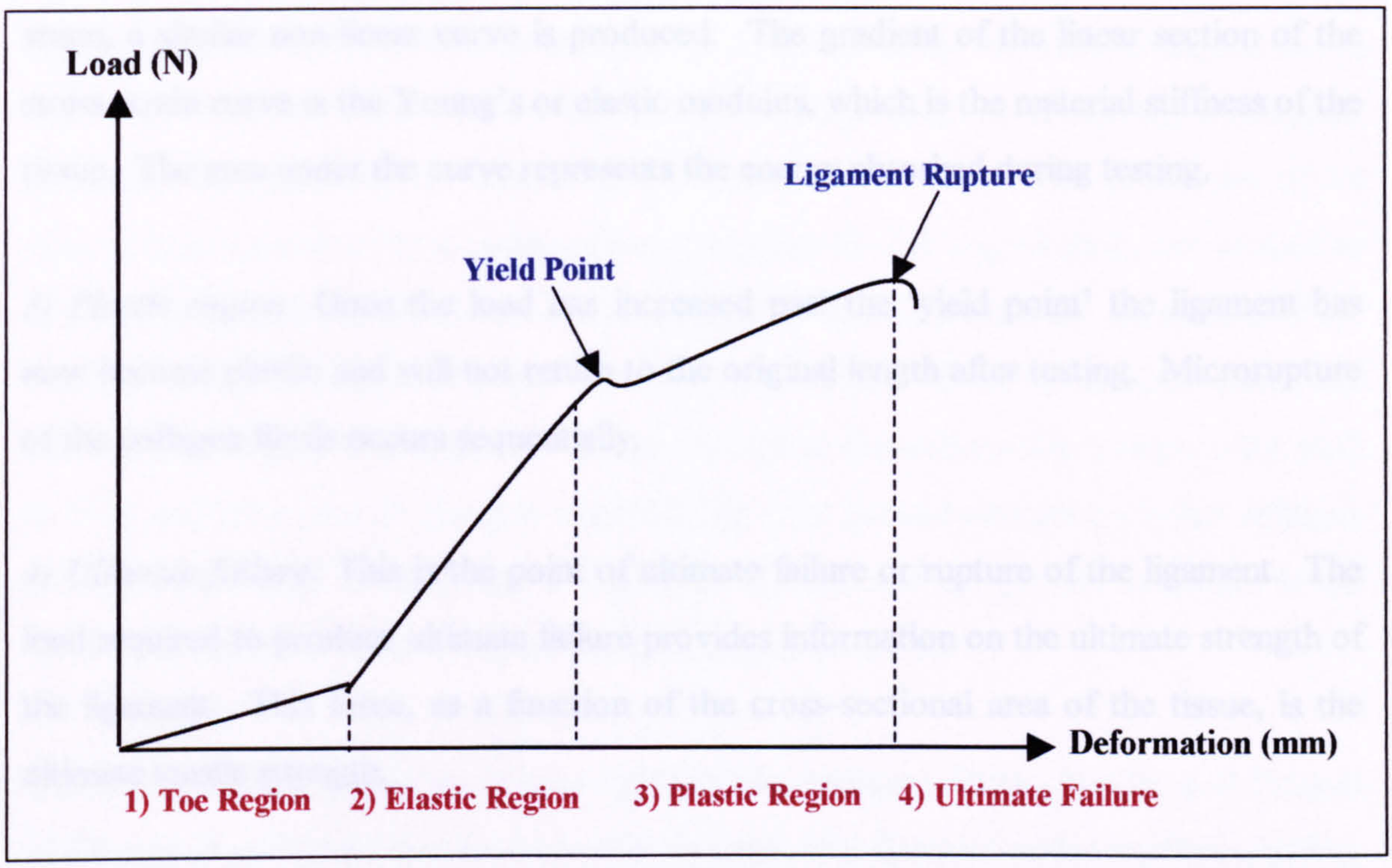


Figure 1.4: A load-deformation curve for loading of a ligament tested to failure. Adapted from Biewener *et al*., (1992), Cabaud *et al*, (1983) and Amis *et al*., (1985).

1) Toe region: This part of the curve relates to the straightening out of the loosely arranged collagen fibres and the orientation of the collagen fibres towards the direction of applied stress. During this initial concave portion of the curve, little force is required to elongate the tissue. This area of the curve gives an indication to the extent of collagen fibril disorganisation and crimp period frequency, and therefore the biomechanical 'laxity' of the ligament (Diamant, Keller et al. 1972; Frank, Amiel et al. 1985). Crimp refers to the three dimensional undulation of the collagen fibril. The presence of crimp reduces the stiffness of the ligament during initial loading and the most likely function of crimp is to decelerate rapid loading thus preventing injury (Frank, Amiel et al. 1985).

2) Elastic or linear region: The ligament at this point is behaving elastically and if the stress is removed at this stage, the ligament would return to its original length. Due to the range of fibre lengths and irregular arrangement within a ligament, at this stage, the fibres will tighten at different extensions (Amis 1985). The gradient of the elastic region provides information on the linear stiffness of the tissue; a structural property. When the

cross-sectional area and length of the tissue is measured and stress is plotted against strain, a similar non-linear curve is produced. The gradient of the linear section of the stress strain curve is the Young's or elastic modulus, which is the material stiffness of the tissue. The area under the curve represents the energy absorbed during testing.

3) Plastic region: Once the load has increased past the 'yield point' the ligament has now become plastic and will not return to the original length after testing. Microrupture of the collagen fibrils occurs sequentially.

4) Ultimate failure: This is the point of ultimate failure or rupture of the ligament. The load required to produce ultimate failure provides information on the ultimate strength of the ligament. This force, as a function of the cross-sectional area of the tissue, is the ultimate tensile strength.

This non-linear behaviour allows ligaments to permit initial joint deformation with minimal resistance. At high force, ligaments become stiffer, providing more resistance to increasing deformation. High load stiffening protects and maintains the integrity of the joint yet the low load compliance allows extension during normal joint movement without damage to the ligament (Frank, Amiel et al. 1985; Biewener 1992).

1.6.2.2 Time Dependent Effects

Ligaments and tendons are viscoelastic materials, as a result of their water and PG content. The viscoelastic behaviour of a ligament determines the time required to produce different elongations of the fibres at different loads. For example, Noyes *et al.*, (1974), showed that when the ACL was experimentally loaded at a fast strain rate, it was stiffer and stronger, showed more elongation and absorbed more energy than a similar ligament loaded at a slower strain rate (Noyes, DeLucas et al. 1974). Creep is a further manifestation of the 'viscosity' properties of ligament material. It is a slow 'flow' process, within the ligament itself, which tends to relieve the structure of load or stress. Creep is thought to involve a combination of water influx, returning collagen crimp, elastin tensile force and increasing collagenous disorganisation (Biewener 1992).

1.6.3 Factors Effecting the Mechanical Properties of Ligaments

Ligaments are composed of many different ECM components, including collagen, elastin and PGs. However the role of the different components in conferring mechanical properties to ligaments is difficult to determine. The mechanical properties of all ligaments are governed by a combination of supermolecular organisation, and molecular organisation and composition. The macromolecular factors include the orientation of the collagen fibres, the extent of crimp, and the tissue shape. Molecular factors include the size and distribution of collagen fibrils, the content of non-collagenous components, such as PGs, and the extent of collagen cross-linking. The general conception is that collagen provides the tensile strength and a degree of stiffness and the ground substance is the 'glue' that maintains tissue integrity (Parry, Craig et al. 1978).

The structural and biochemical properties of the collagen fibres (Nordin and Frankel 1980), can therefore greatly determine the strength of a ligament under loading. Ageing, immobilisation, drugs, systemic diseases and exercise are some factors that can disrupt the collagen and the ground substance of the ligament, and hence reduce its ultimate tensile strength and stiffness (Noyes, DeLucas et al. 1974; Noyes 1977). Those factors most relevant to this study will be discussed in more detail.

Age:

The strength and stiffness of tendons and ligaments have been found to increase with growth and maturation and subsequently decrease slowly with ageing (Noyes and Grood 1976; Woo, Peterson et al. 1990; Woo, Hollis et al. 1991; Becker, Savelberg et al. 1994; Rich and Glisson 1994). This increase in strength and stiffness during maturation is caused by changes in the molecular composition of the collagen. In the ACL and MCL from 2, 12 and 36 month old rabbits the mature intermolecular collagen cross-links were found to increase, whereas collagen synthesis, GAG content and water content decreased, possibly reflecting a reduction in the (re)modelling of the ECM components of the ligament with maturation (Amiel, Kuiper et al. 1991), ultimately increasing the strength and stiffness of the ligament.

This increase in strength and stiffness during maturation is also due to changes in collagen fibril size and distribution (Parry, Craig et al. 1978). In brief, collagen fibrils with a large diameter are thought to have a greater tensile strength than an equivalent mass of collagen arranged in small fibrils as they have more intrafibrillar collagen cross-links. Additionally, those tissues with a bimodal (fibrils of two sizes) distribution of collagen fibrils tend to be stronger and able to withstand high stresses whereas a tissue with unimodal (fibrils of only one size) distribution of collagen fibrils are more suited to resisting low stresses (Davankar, Deane et al. 1996). With maturation the distribution of collagen fibrils has been found to change from a unimodal distribution to a bimodal distribution thus explaining the increased strength and stiffness from birth (Parry, Craig et al. 1978).

The decrease in the strength and stiffness of the ligament with ageing is less well understood, although it is proposed that other factors such as health, diet, disease and level of activity could be the main contributors to this decline with age (Noyes and Grood 1976; Kasperczyk, Rosocha et al. 1991).

Exercise:

Exercise has been shown to increase the mechanical strength and certain biochemical parameters of ligaments and tendons in humans, horses and experimental animals (Woo, Ritter et al. 1980; Woo, Gomez et al. 1981; Simonsen, Klitgaard et al. 1995; Viidik, Nielsen et al. 1996; Wingfield, Amis et al. 2000; Buchanan and Marsh 2001; Buchanan and Marsh 2002). Wingfield *et al.*, (2000) have documented that the linear stiffness, tensile strength and tangent modulus were significantly higher in the CCL of racing Greyhounds compared to the CCL of Rottweilers (a less active breed of dog, more susceptible to CCL rupture). Woo *et al.*, (1981), showed in porcine extensor tendons that exercise resulted in an increase in mechanical strength, collagen content and tendon hypertrophy (Woo, Gomez et al. 1981). Ultrastructural studies on the rat ACL after enforced running, showed increased intracellular activity of the fibroblasts and decreased average fibril diameters, indicative of increased collagen remodelling (Sakuma, Mizuta et al. 1993). Physical exercise has also been shown to prevent the onset of age-related changes to the biomechanical and biochemical properties of ligaments (Viidik, Nielsen et

al. 1996; Nielsen, Skalicky et al. 1998). These studies, in addition to others (Michna and Hartmann 1989; Patterson-Kane, Firth et al. 1998; Cherdchutham, Becker et al. 2001; Kasashima, Smith et al. 2002), demonstrate that exercise can alter the structural properties of collagen, which may ultimately increase tendon and ligament stiffness and strength (Buchanan and Marsh 2002). In a study by Michna *et al.*, (1989) on mice, one week of treadmill exercise was found to increase the mean diameter and cross-sectional area of tendon, and alter the mean fibril diameter distribution (Michna and Hartmann 1989). Similar findings have been documented in the superficial digital flexor tendon (SDFT) of horses (Kasashima, Smith et al. 2002).

The mechanical properties and the ECM composition of ligament and tendon have also been found to be influenced by the type and duration of exercise, and the tendon or ligament type i.e. flexor or extensor tendons, and region i.e. central or peripheral (Woo, Ritter et al. 1980; Woo, Gomez et al. 1981; Patterson-Kane, Firth et al. 1998; Birch, McLaughlin et al. 1999). In a study by Cherdchutham *et al.*, (2001) collagen fibril restructuring was found to be exercise driven in the SDFT of immature foals, with the mean average diameter being found to be bigger in the exercised foals compared to non-exercised controls. Differences were also found in fibril diameters between peripheral and central tendon regions between exercised, restricted exercised and rested foals (Cherdchutham, Becker et al. 2001). Smith *et al.*, (1999) also showed that collagen fibril crimp angle and length were reduced in the central core of the SDFT with exercise and age compared to the peripheral regions. This only correlated in long-term exercised older horses, but not in short-term exercised or younger horses. Smaller fibrils were found in older, exercised horses, but were not consistent with new collagen formation suggesting that they were present as a result of disassembly of larger fibrils. Collagen type III, GAG and COMP content were also increased in the older, exercised horses suggesting that older exercised horses are more susceptible to SDFT injury than younger, exercised horses (Smith, Birch et al. 1999).

Studies examining the effect of exercise and age on the biomechanics and ECM metabolism of the MPICL have not been reported.

1.7 BONE FORMATION AND REMODELLING

Since bone is a more dynamic tissue than ligament and cartilage, and this study aims to assess the response of bone to exercise, it is fitting that this connective tissue is discussed in more detail.

1.7.1 Embryonic Bone Formation

Embryonic bone development can be divided into intramembranous and endochondral ossification:

a) Intramembranous Ossification

Intramembranous ossification results in the flat bones of the skeleton (e.g. cranium, scapula), and develops directly from the mesenchyme. Mesenchymal cells within a richly vascularised area of connective tissue differentiate into osteoblasts which synthesise the primary bone matrix which is subsequently mineralised. As this process progresses, blood vessels are trapped and later form haematopoietic bone marrow. In the adult this primary bone is remodelled and replaced by inner and outer layers of cortical bone sandwiching a layer of trabecular bone (Marieb 1995). Intramembranal bone has primarily a protective function and is of little metabolic importance, and consequently will not be discussed further.

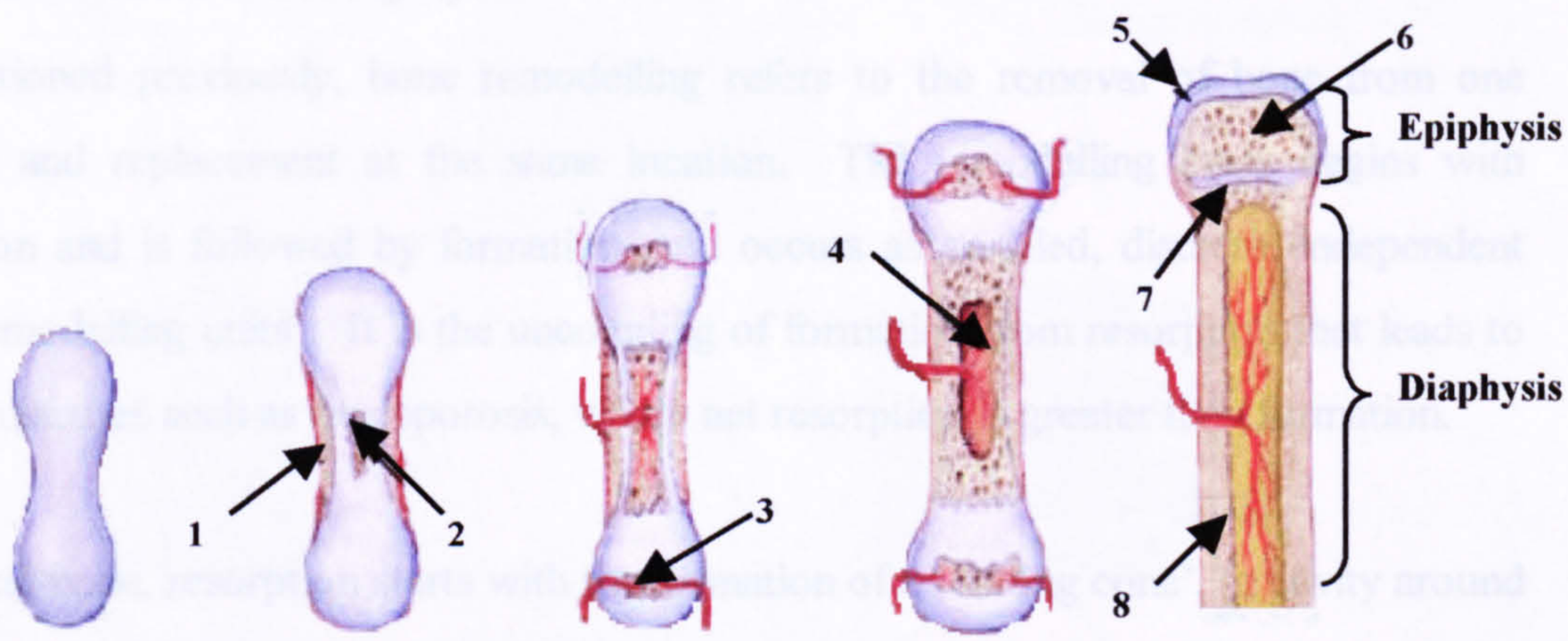
b) Endochondral Ossification

Endochondral bone formation is much more complex than intramembranous ossification and is summarised in Figure 1.5. Essentially this process involves the replacement, with bone, of an embryonic hyaline cartilage rudiment produced by the mesenchymally derived chondroblasts. These chondroblasts become chondrocytes as they become embedded within the cartilage, and subsequently de-differentiate to osteoblasts (Marieb 1995). The calcified matrix is then resorbed by osteoclasts (derived from haematopoietic cells) and replaced by 'woven' bone produced by osteoblasts to form the primary spongiosa (an irregular trabecular network), which is later replaced by the secondary spongiosa consisting of organised trabeculae of lamellar bone.

The perichondral bone of the midshaft is formed by intramembranous ossification on the surface of the cartilage rudiment by cells derived from the perichondrium (later the periosteum). The resulting collar of bone is invaded by osteoclasts, which resorb the central calcified cartilage, allowing the subsequent infiltration by blood vessels. This area is subsequently filled with cells, which differentiate to form either osteoblasts or haematopoietic marrow tissue. The former produce a network of irregular trabeculae, a proportion of which are later resorbed to leave a marrow cavity (Marieb 1995).

The primary, woven bone mentioned above, is characteristic of the rapidly formed bone produced during periods of growth and fracture repair. The collagen fibres are produced as disorganised fibre bundles and consequently mineralisation of the matrix is also disorganised. This woven bone is ultimately replaced by adult, cortical (lamellar) bone. This consists of sheets of highly organised collagenous matrix, with the fibres laid down in a preferential orientation. The mineralisation occurs as a controlled front following soon after matrix formation, resulting in the orderly deposition of the mineral crystals. This controlled formation of cortical bone culminates in a mechanically specialised structure (Marieb 1995).

Growing bones possesses a secondary ossification centre at the epiphyses. A narrow cartilaginous epiphyseal growth plate remains between the epiphysis and the diaphysis, allowing growth to continue until maturation is reached at which point the ossification centres unite.



A Cartilage bone rudiment

B Initial deposition of a collar of perichondral bone, and central calcification of the cartilage.

C Development of secondary ossification centres in the epiphysis and growth of the endochondral and perichondral bone towards the epiphyses.

D Resorption of endochondral bone and replacement by a marrow cavity. Formation of growth plate.

E End of growth with fusion of the two centres of ossification. Enlargement of marrow cavity.

1 Bone Collar

2 Primary Ossification Centre

3 Primary Ossification Centre

4 Medullary Cavity

5 Articular Cartilage

6 Endochondral (trabecular) Bone

7 Epiphyseal Plate

8 Perichondral (cortical) Bone

Figure 1.5: Stages in the endochondral ossification occurring in a long bone. Adapted from Marieb *et al*., (1995).

1.7.2 Bone Remodelling

1.7.2.1 Bone Remodelling Cycle

As mentioned previously, bone remodelling refers to the removal of bone from one location and replacement at the same location. The remodelling cycle begins with resorption and is followed by formation, and occurs as coupled, discrete, independent 'bone remodelling units'. It is the uncoupling of formation from resorption that leads to skeletal diseases such as osteoporosis, where net resorption is greater than formation.

In cortical bone, resorption starts with the formation of a 'cutting cone', (a cavity around a capillary in the old bone) by bone resorbing osteoclasts. The newly exposed bone surfaces are then invaded by osteoblast precursor cells (mononuclear cells), which subsequently mature into osteoblasts, which begin to lay down unmineralised bone matrix termed osteoid, which upon mineralisation form thin sheets called lamellae (Figure 1.6). Successive cycles of resorption and formation result in characteristic osteons or Haversian system (Figure 1.7). This lamellar bone is generally thought to consist of layers of mineralised collagen with the fibres laid down at differing angles between layers, resulting in a 'plywood' arrangement. The name of this type of bone is derived from the lamellar effect observed under polarised light microscopy (Marieb 1995).

The formation of bone in the trabecular region also results in a lamellar system, but the layers are flat and parallel, due to trabecular bone having no osteons. Due to its greater surface area, trabecular bone is turned over more rapidly than cortical bone. In humans, the trabecular bone, which represent about 20% of skeletal mass, makes up 80% of the remodelling of bone, while the cortical bone, which represents 80% of the bone, makes up only 20% of the remodelling (Christiansen, Hassager et al. 1998). This may give some indication as to the reason for stress fracture to occur in bones with a higher percentage of trabecular bone, such as the Cr and C3.

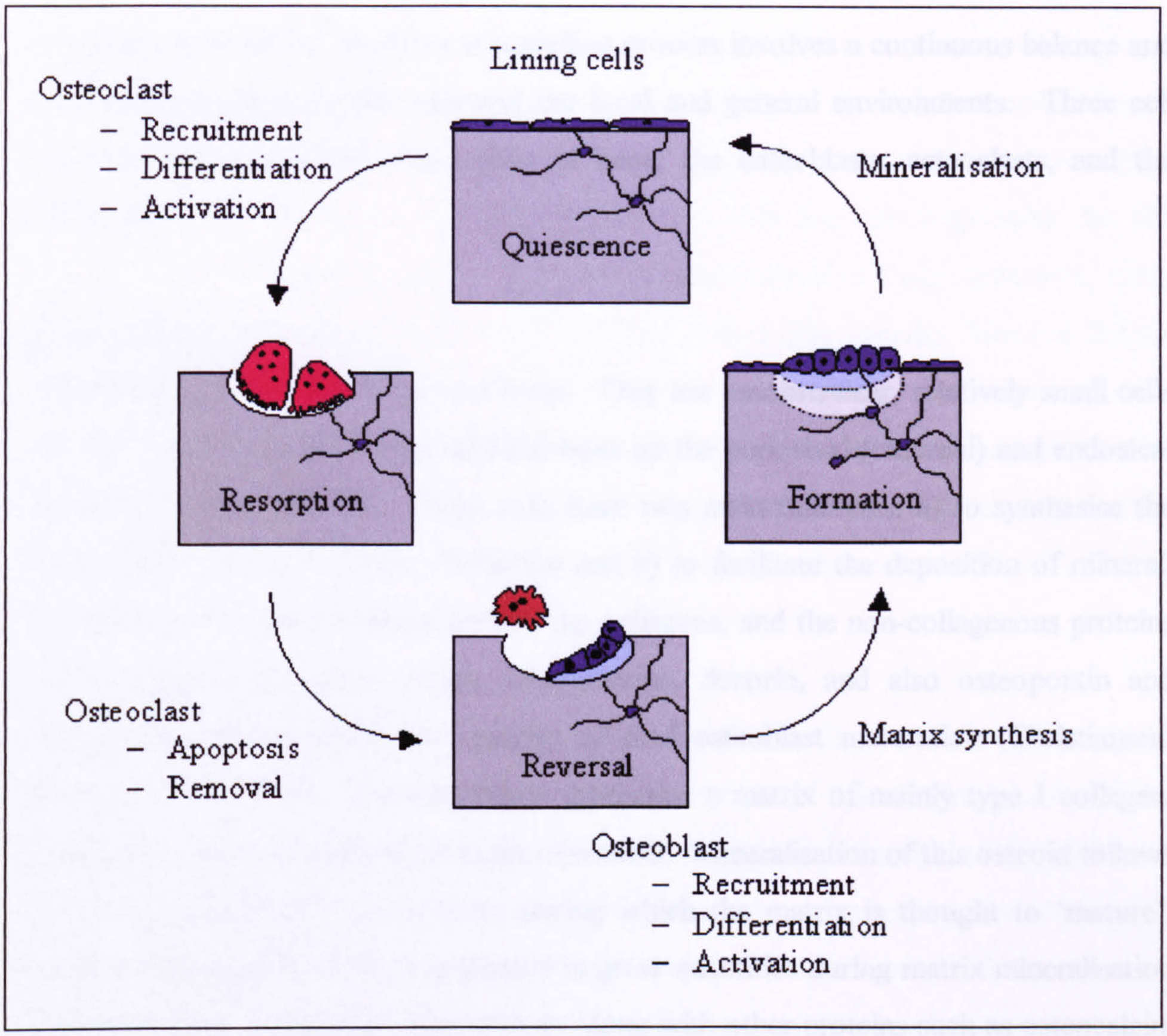


Figure 1.6: The bone remodelling cycle.
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Accessed: 20th June 2004.

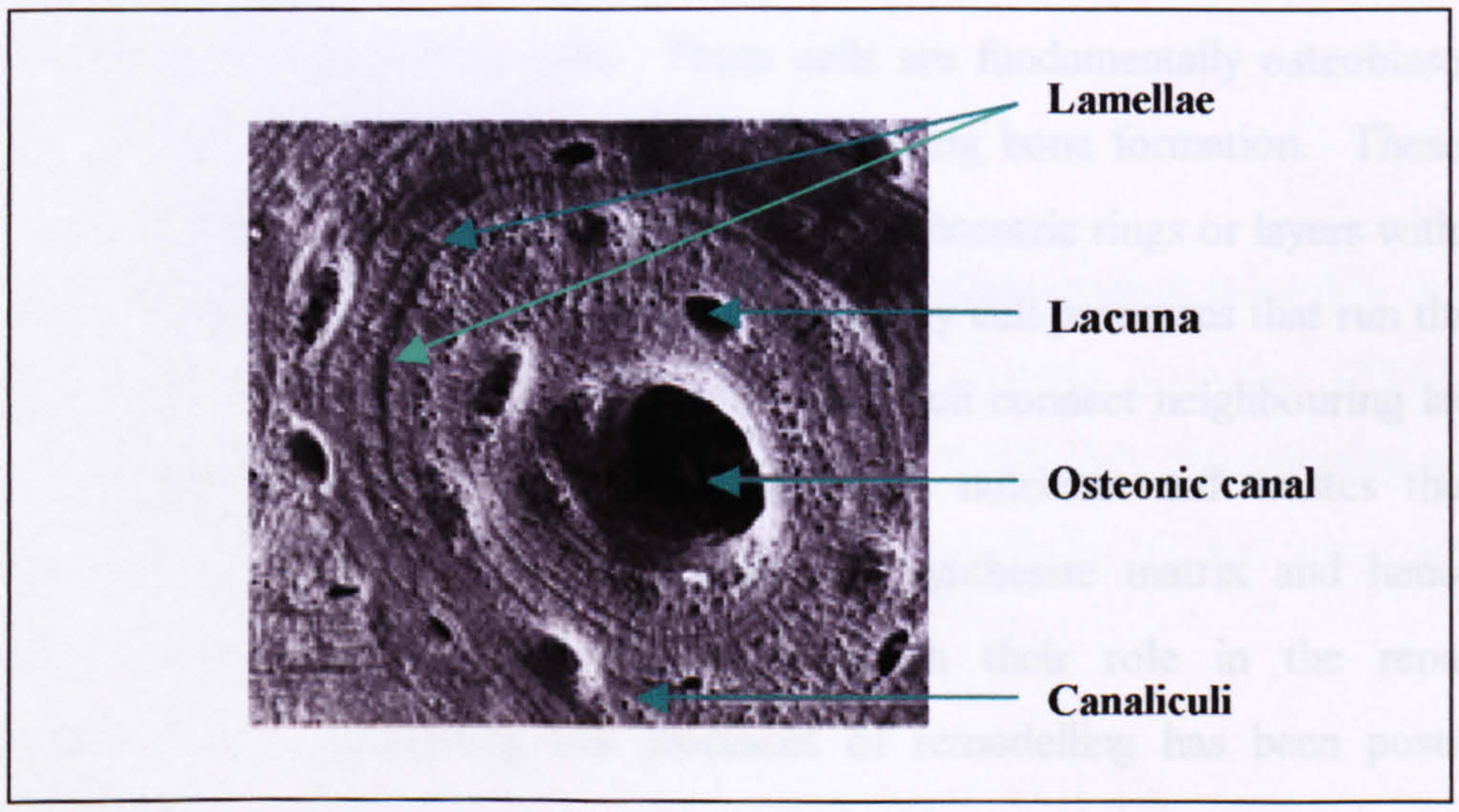


Figure 1.7: Electron microscopy of an osteon in cortical bone, illustrating the sheets of lamellae (Kessel and Kardon 1979).

As evident from above, the bone remodelling process involves a continuous balance and communication between the cells and the local and general environments. Three cell types are involved in the remodelling of bone, the osteoblasts, osteoclasts, and the osteocytes.

a) Osteoblasts:

Osteoblasts are cells that form new bone. They are mononuclear, relatively small cells and are often found as a single cuboidal layer on the periosteal (external) and endosteal (internal) surfaces of bone. These cells have two main functions; a) to synthesise the characteristic proteins of bone formation and b) to facilitate the deposition of mineral. The proteins of bone formation include the collagens, and the non-collagenous proteins such as bone sialoprotein, matrix GLA-protein, decorin, and also osteopontin and osteocalcin, both of which are markers of final osteoblast maturation (Christiansen, Hassager et al. 1998). The osteoblasts synthesise a matrix of mainly type I collagen, initially at a rapid rate, forming a seam of osteoid. Mineralisation of this osteoid follows after a lag period of 5 to 10 days, during which the matrix is thought to 'mature'. Alkaline phosphatase (ALP) is produced in great quantities during matrix mineralisation in which it plays a key role. This enzyme along with other proteins such as osteocalcin, appear in blood and urine and can be used as measures of bone formation.

b) Osteocytes:

Osteocytes are bone maintaining cells. These cells are fundamentally osteoblasts that have been 'trapped' in the calcifying matrix front during bone formation. These cells reside in little hollows called lacunae that are seen in concentric rings or layers within the bone matrix. Neighbouring osteocytes are connected by cell processes that run through tiny tunnels called canaliculi (Figure 1.7). The canaliculi connect neighbouring lacunae and the cell processes running through them move nutrients and wastes that the osteocytes need or produce. Osteocytes do not synthesise matrix and hence are metabolically less active than osteoblasts, although their role in the repair of microdamage, and in modulating the processes of remodelling has been postulated (Lanyon 1992b).

c) Osteoclasts:

Osteoclasts are bone matrix degrading cells. These cells are formed from the fusion of monocyte precursors that are derived from the pluripotent haematopoietic stem cells of the bone marrow. They are large multinucleated cells and are responsible for the resorption of bone, and are readily identified by their extensive Golgi apparatus, many mitochondria and characteristic ruffled border. The ruffled border forms a limited portion of the membrane apposed to the bone surface and is contained within a peripheral membrane 'clear zone'. This actin rich zone both attaches the osteoclast to the surface via integrin receptors (probably vitronectin) and forms a tight seal around the resorption space. It is in this space that resorption occurs via the ruffled border (Baron 1993).

The initial phase of resorption is the digestion of the non-collagenous link between the mineral crystals and the collagen, allowing the dissolution of the mineral, which is aided by the presence of calcium chelating anions such as citrate. This is then closely followed by the degradation of the organic matrix by metalloproteinases and lysosomal enzymes such as cathepsin B or K released across the ruffled border. One of these enzymes, tartrate resistant acid phosphatase (TRAP), is widely used as a marker for osteoclasts due to its prolific synthesis (Minkin 1982). After up to seven weeks of activity the osteoclasts migrate to the bone marrow and die (Lian and Stein 1999).

1.7.2.2 The Regulation of Bone Remodelling

The control of bone remodelling is an extremely complex process involving systemic hormones and vitamins such as vitamin D and parathyroid hormone (PTH), and autocrine and paracrine growth factors and cytokines, particularly transforming growth factor-beta (TGF-β), prostaglandin E_2 (PGE_2), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α).

Systemic Control:

PTH, vitamin D3 and calcitonin are the main systemic regulators of bone remodelling. These factors have effects on the actions of each other as well as on the bone cells, so an

imbalance of one hormone can have a cascade effect. Such an occurrence may result in the uncoupling of the bone resorption/formation cycle manifesting skeletal diseases such as hyperparathyroidism and osteoporosis.

a) PTH

PTH, an 84-amino acid peptide hormone synthesized by the parathyroid glands, is an important modulator of calcium homeostasis. PTH secretion is stimulated by low plasma ionic calcium concentration, and other divalent cations, such as magnesium, strontium, and manganese. In bone, PTH causes an increase in osteoclastic bone resorption and bone remodelling. PTH mediates its effect by binding to extracellular membrane-bound receptors that activate an intracellular adenylate cyclase-dependent signal transduction system. When activated, adenylate cyclase causes an increase in intracellular cyclic adenosine monophosphate (AMP). As osteoclasts do not have PTH receptors, their response to PTH is dependent on PTH binding to the membrane-bound receptors on osteoblasts, which then indirectly mediate the osteoclastic resorption through secondary coupling factors. Through this mechanism, PTH can modulate both bone resorption and bone formation. PTH simultaneously elevates the serum ionised calcium level by the release of calcium ions from the bone matrix and promotes bone remodelling through the activation of osteoblasts and osteoclasts (McSheehy and Chambers 1986).

PTH is also the major controlling factor of the level of the active metabolite of vitamin D, $1,25(OH)_2D_3$ (calcitriol or vitamin D3) by its effects on the activity of renal 1α-hydroxylase enzyme (Anderson 1990).

b) Vitamin D3

Vitamin D3, like PTH, is also involved in the control of calcium homeostasis. The receptors for vitamin D3 are regulated by PTH, oestrogen, glucocorticosteroids and are also self-regulated. Several allelic variants in the gene for the vitamin D3 receptor have been described (Kelly, Hopper et al. 1991; Morrison, Qi et al. 1994). Morrison *et al.*, (1994), have reported an association between BMD and the specific allelic variants in the gene encoding the vitamin D3 receptor ($1,25(OH)_2D_3$) and suggested that this genetic marker could be used as a risk factor for osteoporosis (Morrison, Qi et al. 1994).

Vitamin D3 influences a range of osteoblastic functions. Effects on cultured osteoblasts include the stimulation of ALP (Farley, Hall et al. 1994) and osteocalcin production (Beresford, Gallagher et al. 1984), the modulation of type I collagen production, and the release of cytokines and matrix proteins (Owen, Aronow et al. 1991). Vitamin D3 additionally stimulates bone resorption by increasing the activity of existing osteoclasts and by promoting the differentiation of osteoclast precursors into mature multinucleated osteoclasts.

c) Calcitonin

Calcitonin is the principal inhibitor of bone resorption, and the only major hormone commonly accepted to have a direct effect on osteoclasts. Its actions are mediated by concomitant changes in intracellular cyclic AMP. Its secretion from the C cells of the thyroid is stimulated by high serum calcium and vitamin D3, which, in addition, may induce formation of receptors (Takahashi, Akatsu et al. 1988). The physiological importance of calcitonin remains unclear, as it does not significantly affect serum calcium levels, and its actions are relatively short-lived. Calcitonin may therefore serve to 'protect' the skeleton from resorptive hormones, or respond to short term requirements of calcium homeostasis.

Autocrine/ Paracrine Control:

It is now accepted that the major actions of hormones are mediated through local factors such as cytokines and growth factors for both formation and resorption. Cytokines and growth factors are important mediators of cell-to-cell communication. They can also mediate the effects of many systemic hormones locally. Acting in autocrine and paracrine ways, they can promote cell proliferation, cell differentiation, bone formation, and bone resorption.

a) Cytokines:

IL-1β and TNF-α are the main cytokines involved in bone resorption. Both these cytokines have their direct action on the osteoblast, and result in synergistic stimulatory effects on the osteoclast (Pierce, Lindskog et al. 1991). IL-1 has been shown to

stimulate the production of IL-6 by the osteoblast, and since IL-6 appears to be involved in the same pathway as IL-1, it is thought that it is an important mediator of IL-1 action.

Interferon-γ (IFN-γ) is thought to be an antagonist to IL-1β and TNF-α, and other factors, which promote bone formation including transforming growth factor-α (TGF-α) and insulin growth factors (IGFs).

b) Growth factors:

The IGFs directly affect collagen synthesis and promote osteoblast replication. The importance of this cytokine on the maintenance of bone mass and possibly bone strength, has led to investigations into its therapeutic use, although indirectly by systemic factors such as PTH which locally stimulate IGF production (Canalis 1993).

TGF-β is an important growth factor involved in bone formation and resorption. It has both stimulatory and inhibitory effects on bone resorption and as a consequence is thought to be a mediator of the coupling between formation and resorption. It also enhances bone collagen synthesis, and is thought to be important for normal matrix production. TGF-β is secreted in an inactive form with activation occurring by local changes in the pH by bone resorbing agents, such as plasmin, and by autostimulation. As a consequence the levels, activity, and response to this growth factor are critical for normal bone maintenance (Canalis 1993).

c) Other regulators:

The prostaglandins, especially PGE_2, are also important local hormones in the maintenance of normal bone turnover. They are produced by osteoblasts, stimulate osteoclastic bone resorption, and are thought to play a central role in the skeletal response to changes in its mechanical environment (Lanyon 1992a). Many factors affect prostaglandin synthesis, most notably PTH and oestrogen, which increase and decrease production respectively.

1.8 THE MINERALISATION OF BONE

The mineralisation of bone refers to the ordered deposition of apatite on a type I collagen matrix. The mature bone apatite crystals are always deposited such that they lie parallel to the axis of the collagen fibril (Boskey 1999). The process of mineralisation is complex, involving the initiation of mineralisation and mineral growth.

Initiation:

The initiation of mineralisation is often described as a three-step process comprising of an increase in ion-products, provision of nucleation sites and removal of inhibitors (Boskey 1992). Two theories of the initiation of mineralisation have been proposed; the nucleation theory and the matrix vesicle theory, both of which encapsulate the three-step process.

The nucleation theory proposes that the type I collagen fibril is the major site of crystal nucleation where calcium phosphate crystals are deposited in the gap regions of the highly organised collagen fibrils; the gap regions providing a specific micro-environment for nucleation. Originally it was believed that the initiation of bone mineralisation is mainly an extracellular, biochemical process where the presence of collagens, certain GAGs or lipids could trigger the initiation of calcification (Wuthier 1968; Wuthier 1969; Wuthier and Irving 1969; Shuttleworth 1972).

The matrix vesicle theory arose when cellular activity was recognised to be needed for the mineralisation of bone. Shapiro *et al.*, (1969) found that the mitochondria of bone-forming cells produced a local rise in the levels of mineral ions, and also a matrix, which was capable of being mineralised (Shapiro and Greenspan 1969). In addition, the mitochondria of columnar and hypertrophic zones of growth plate cells were found to contain more enzymes associated with calcification, such as ALP, than did those of the resting zone (Arsenis 1972). Although the presence of matrix vesicles in certain calcifying tissues has been known for many years, the exact role of these structures is still unclear (Boskey 1999).

However, both theories are not mutually exclusive, with both processes probably occuring depending upon the tissue site and stage of development. In mature bone,

which has a highly organised matrix, initiation of mineralisation by nucleation probably occurs, where as in embryonic or rapidly growing bone, where there is less organisation between the collagen fibrils and the mineral, matrix vesicle initiation predominates (Boskey 1999).

Mineral Growth:

Whether by matrix vesicle mediated, or collagen mediated mineral initiation, subsequent growth of the crystal 'seeds' is a physiochemical process depending upon the local environment. Matrix vesicles are only involved in the initial stage of the mineralisation process (Anderson 1989).

Once formed, crystals grow rapidly. Mineral crystals have been variously described as needle or plate-like, but it is generally accepted that they are thin, plate-like crystals, which soon 'out-grow' the gap zone. Several studies have suggested that the gap zones of the collagen molecules are aligned so as to form a continuous channel within the fibrils. The organisation of collagen in this way would provide space for subsequent mineral growth (Boskey 1999). Crystals growing from various sites within the collagen fibril eventually coalesce to form sheets of mineral between layers of collagen molecules.

1.9 COLLAGEN

Since the major component of bone, cartilage and ligament is collagen, it is appropriate that the collagen superfamily is discussed in detail.

1.9.1 The Collagen Types

The collagens, a specialised group of proteins, are essential components of all connective tissues, whose diverse structure is reflected by the wide variety of functions. Collagen is present to some extent in nearly all organs and tissues and currently 27 genetically distinct types have been identified. For clarity the most documented collagen types can be categorised into four main groups depending on their structure and/or their function (Prockop and Kivirikko 1995; Bailey and Paul 1997):

a) Fibrous collagens, e.g. types I, II and III, which accounts for 85-90% of the collagens in the body, and minor collagen types V and XI

b) Non-fibrous collagens, e.g. types IV, VIII and X

c) Filamentous collagens, e.g. types VI and VII

d) Fibril associated collagens, e.g. types IX, XII, XIV, XV, XVI and XIX.

In general, the collagens are comprised of a right handed triple helix formed from three left handed polypeptide chains which are comprised of repeating triplets of –Gly-X-Y-, in which the Glysine residues are in the centre of the helix and X and Y are on the surface (Kielty, Hopkinson et al. 1993). In about one-third of cases, X is a proline and Y is a hydroxyproline. The presence of hydroxyproline is essential to stabilise the triple helix and is an almost unique characteristic of collagen molecules (Rossert and de Crombrugghe 1996). In addition, most collagens possess short non-triple helical amino acid sequence regions, which in the fibrous collagens are located at the ends of the triple helix.

a) Fibrous Collagens:

The fibrous collagens are by far the most abundant collagen type in the body and are defined by their ability to assemble in a quarter-staggered array to form fibrils with diameters between 25 and 400nm. The main collagen types of this group are types I-III, and are generally found as heteropolymers with types V and XI. Type I collagen, quantitatively the most important, forms thick fibrils and is mainly found in bone, tendon, ligament and skin. There is also some evidence that the type I fibril has a core of some other material than type I, such as type III (Franc 1993). Type II fibrils are narrower and are found mainly in cartilage and the vitreous humour. Type III is mainly found in tissues with some elasticity such as skin and lung and has been identified at the repair site of injured tissue, where it can form rapid cross-links and aid in the stabilisation of the repair site (Liu, Yang et al. 1995). Type V, though quantitatively minor, is always found in close association with type I and III, and may form cross-links with type I molecules. Type XI is more tissue specific, accounting for 8-10% of the collagen in cartilage (Kielty, Hopkinson et al. 1993).

The main function of these collagens is to provide the tensile strength to the tissue, the importance of which is underlined when genetic defects involving these proteins, such as osteogenesis imperfecta (brittle bone) and Ehlers Danlos syndrome (extensible skin), result in severe abnormalities of the tissue involved.

b) Non-fibrous Collagens:

Collagen types IV, VII, VIII and X are grouped together as non-fibrous collagens although their macrostructural organisation varies considerably. Type IV, the most abundant non-fibrous collagen, possesses a long triple helix (400nm) which lacks the charge profile required for the formation of quarter staggered fibres, and in contrast to the fibrous collagens retains its terminal globular domains in the aggregated structure. The 3-dimensional structure of type IV collagen, often described as a 'chicken-wire' structure, forms the basis of basement membranes. Types VIII and X are thought to form the hexagonal lattices of the Decemet's membrane (in the cornea) and calcifying cartilage respectively (Bailey and Paul 1997).

c) Filamentous collagens:

Type VI collagen has been shown to form loosely packed filamentous structures which have a repeating pattern of about 100nm and is widely distributed as a minor component of many different collagenous tissues, such as, cartilage, skin, tendon and bone, but the function of these fibres has not been elucidated. It has been suggested that they play a role in the spacing and alignment of other fibres in tissues, for example, the highly organised type I fibres in the cornea (Bailey and Paul 1997). Type VII collagen acts as an anchoring collagen, attaching basement membranes of different collagen types.

d) Fibril Associated Collagens:

The collagens within this group are often termed FACIT (Fibril Associated Collagen with Interrupted Triple helices) collagens and include type IX, XII, XIV, XV, XVI and XIX. As this term suggests, they are characterised by short triple helical domains interrupted by short non-collagenous sequences and generally associate themselves with fibres of types I and II (Kielty, Hopkinson et al. 1993). Type IX is covalently linked to type II and is found in cartilage (Prockop and Kivirikko 1995). Types XII, XIV and XVI are similar in structure to type IX and are found in type I rich tissues, and may also be present in cartilagenous tissues (Ayad, Boot-Handford et al. 1994). Type XIX is a relatively new member of this group and shows structural similarities to the other FACIT collagens.

Although various collagen types exist, much of the information on the biochemical and biophysical properties, cross-linking, and synthesis of collagens has been obtained on collagen type I, so it is with reference to this collagen type that the following will be made.

1.9.2 Structure and Synthesis of Type I Collagen

a) Collagen Structure

The characteristic feature of a type I collagen molecule is its triple stranded helical structure (polyproline helix), which is typically composed of two α1(I) chains and one α2(I) chain (heterotrimer), with each α chain consisting of approximately 1000 amino acid residues, often with 330 –Gly-X-Y- repeats per chain. A very small number of type

I collagen molecules can also be formed by three α1(I) chains (homotrimer). The α1(I) and α2(I) propeptides are genetically distinct and are encoded by the genes COL1A1 (18kb in length) and COL1A2 (40kb in length) respectively (von der Mark 1999).

Type I collagen is synthesised as procollagen, which consists of a long triple helical domain, about 300nm long and 1.5nm in diameter, preceded by a short N terminal propeptide (15kd) and followed by a longer C terminal propeptide (30kd). These propeptides are cleaved post-secretion into the extracellular matrix. The resulting tropocollagen is mainly a triple stranded helix with small globular regions, telopeptides, at the N and C terminal. Once the propeptides are excised, type I collagen fibrils form by spontaneous aggregation of the tropocollagens, which are staggered by approximately 67nm to form the characteristic quarter staggered array (refer to Figure 1.8) (Keilty et al., 1993; Lodish et al., 1995).

Electron micrographs of stained collagen fibrils indicate that tropocollagens are separated by approximately 40nm gaps, these gaps are important in enabling collagen to become cross-linked after the fibril forms. These gaps also play a role in bone formation and mineralisation as previously mentioned (Stryer 1988; Rossert and de Crombrugghe 1996).

b) Collagen Synthesis

The collagen molecule undergoes several stages of intracellular and extracellular processing, beginning life as a native pre-propeptide, secreted as procollagen and finally aggregated as tropocollagen. Many co- and post-translational modifications occur during these processes to ensure the correct structure and function of the collagen. The main synthesis events are summarised in Figure 1.8 and Table 1.2. Only some of the characteristic processes of collagen biosynthesis will be discussed here as this topic has been reviewed elsewhere (Kielty, Hopkinson et al. 1993; Berisio, Vitagliano et al. 2002).

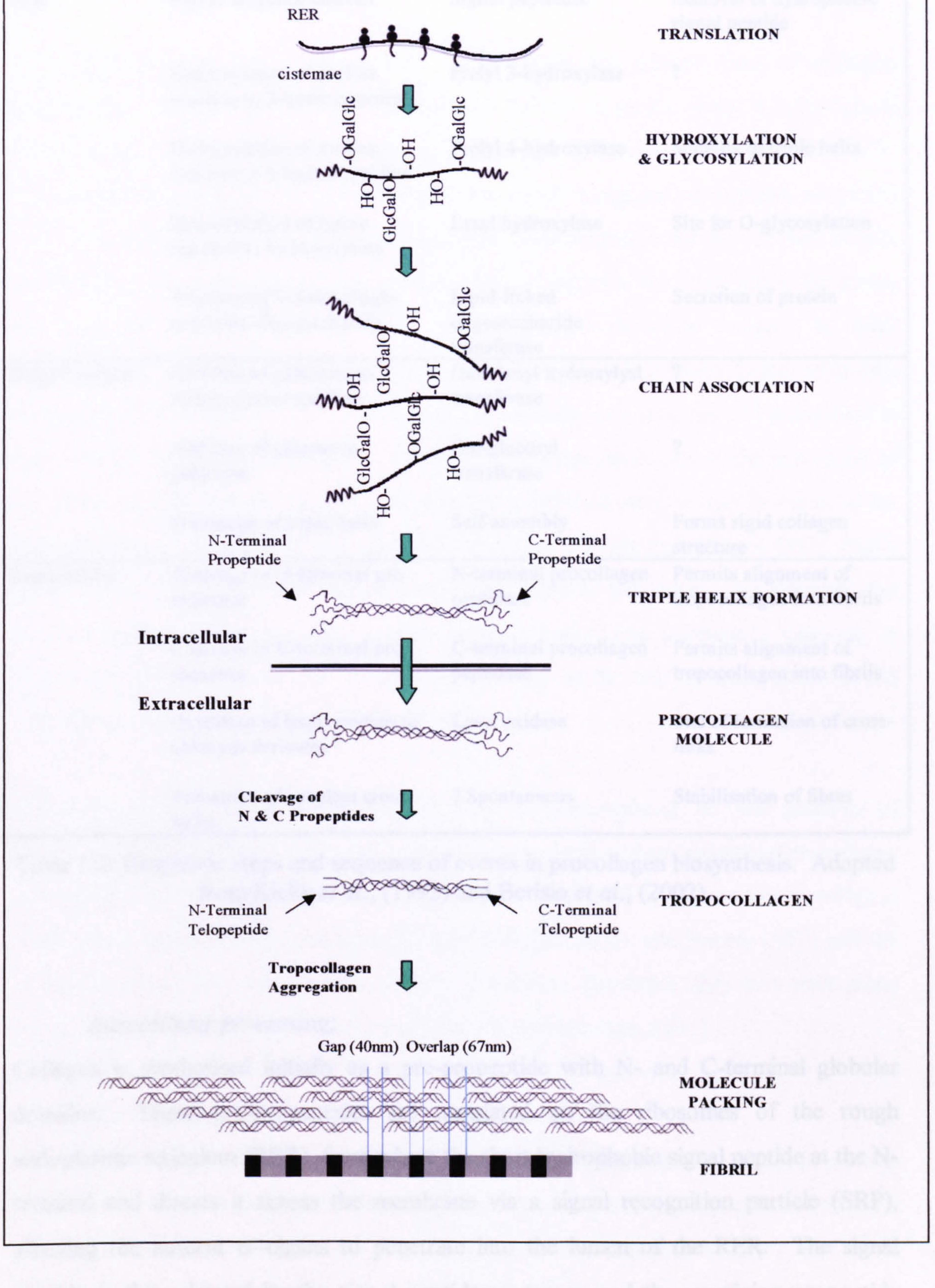


Figure 1.8: Intracellular and extracellular synthesis and processing of the collagen triple helix. Adapted from Kielty *et al.*, (1993).

Location	Major Events	Enzyme	Function
RER	Signal sequence cleaved	Signal peptidase	Removal of hydrophobic signal peptide
	Hydroxylation of proline residues to 3-hydroxyproline	Prolyl 3-hydroxylase	?
	Hydroxylation of proline residues to 4-hydroxyproline	Prolyl 4-hydroxylase	Stability of triple helix
	Hydroxylation of lysine residues to hydroxylysine	Lysyl hydroxylase	Site for O-glycosylation
	Addition of N-linked high-mannose oligosaccharide	Lipid-linked oligosaccharide transferase	Secretion of protein
Golgi Complex	Addition of galactose to hydroxylysine residues	Galactosyl hydroxylysl transferase	?
	Addition of glucose to galactose	Gal-glucosyl transferase	?
	Formation of triple helix	Self-assembly	Forms rigid collagen structure
Extracellular	Cleavage of N-terminal pro sequence	N-terminal procollagen peptidase	Permits alignment of tropocollagen into fibrils
	Cleavage of C-terminal pro sequence	C-terminal procollagen peptidase	Permits alignment of tropocollagen into fibrils
	Oxidation of lysyl residues to aldehyde derivates	Lysyl oxidase	Site of formation of cross-links
	Formation of covalent cross-links	? Spontaneous	Stabilisation of fibres

Table 1.2: Enzymatic steps and sequence of events in procollagen biosynthesis. Adapted from Kielty *et al.*, (1993) and Berisio *et al.*, (2002).

Intracellular processing:

Collagen is synthesised initially as a pre-propeptide with N- and C-terminal globular domains. These pre-propeptides are translated on the ribosomes of the rough endoplasmic reticulum (RER), from where the short hydrophobic signal peptide at the N-terminal end directs it across the membrane via a signal recognition particle (SRP), allowing the nascent α–chains to penetrate into the lumen of the RER. The signal peptide is then cleaved by the signal peptidase enzyme and the remaining propeptide undergoes enzymatic post-translational modification in the RER before triple helix formation, primarily the hydroxylation of lysine, and proline and the subsequent

glycosylation of hydroxylysine residues (Figure 1.8 and Table 1.2) (Kielty, Hopkinson et al. 1993; von der Mark 1999).

Hydroxylation and, to a lesser extent, glycosylation are critical steps of collagen biosynthesis. Proline residues are hydroxylated by two enzymes; prolyl 4-hydroxylase, which forms 4-hydroxyproline residues, and prolyl 3-hydroxylase, which forms 3-hydroxyproline residues. Prolyl 4-hydroxylase requires oxoglutarate, oxygen, ascorbate and ferric ions as cofactors. Ascorbate is an essential co-factor for the reaction as it is thought to act as an uncoupled oxygen acceptor and recycles the ferric ion at the active site back to its active ferrous state. Hydroxylation of lysine residues to form hydroxylysine is catalysed by the enzyme lysyl hydroxylase to form 5-hydroxylysine. Hydroxylysine is important for the attachment of carbohydrate residues and is involved in intra and intermolecular cross-links. There has been speculation about the specificity of lysyl hydroxylase, as there is some evidence that a different enzyme may be required for hydroxylation of telopeptide and triple helical lysines (Royce and Barnes 1985; Bank, Robins et al. 1999). Several circumstantial facts support this theory, for example, the level of hydroxylation may differ greatly between helical and telopeptide residues, in addition to the absence of the X-Lys-Gly sequence in the telopeptides, which is the recognition site for this enzyme (Miller 1976).

The hydroxylysine residues can subsequently be glycosylated by the addition of galactose. This process is catalysed by the enzymes, hydroxylysyl galactosyl transferase and galactosyl hydroxylysyl glucosyl transferase. The level of glycosylation within a tissue varies between tissue and collagen type and age (Royce and Barnes 1977) and the biological role of the carbohydrate residues is unclear. However, they may have some role in fibril organisation and diameter (Kielty, Hopkinson et al. 1993).

Helix formation:

Assembly of the procollagen chains to form triple helical molecules is a complex process involving the C-terminal ends of the propeptide chains and is thought to proceed through a series of events involving chain alignment, nucleation and propagation. The chain alignment is thought to be initiated by attachment of the signal propeptides of the nascent α–chains to the RER, which when near full elongation, can fold in a C to N terminal

direction. The association of the α–chains is thought to be initiated by noncovalent interactions at the C-terminal regions, with helical conformation arising largely from the steric repulsion between the proline and hydroxyproline residues. The rigid five-membered imino acid (pyrrolidine) rings of proline and hydroxyproline are situated on the exterior of the triple helix; the fixed angle of the C-N peptidyl-proline or peptidyl-hydroxyproline bond enables each polypeptide chain to fold into the polyproline helix. The rigidity of the imino acid rings also has a stabilising effect on the collagen molecule and limits rotation about the peptide C-N bond (Kielty, Hopkinson et al. 1993).

The triple helix is further stabilised by an extensive network of hydrogen bonds. Glycine, the smallest amino acid, can pack tightly at the interior position of the helix, where it provides NH-groups for hydrogen bonding to a peptide carbonyl (C=O) group in an adjacent polypeptide, thus helping to hold the three chains together. Hydroxyproline residues can also create additional hydrogen bonds, further increasing the stability of the helix.

Secretion and fibril formation:

Once triple helix folding is complete, procollagen is secreted via the Golgi complex, into the extracellular matrix, by exocytosis. Procollagens are unable to aggregate and form collagen fibrils until the terminal propeptides are cleaved by extracellular proteinases. This post secretion processing of fibrillar collagens prevents undesirable intracellular helix formation. Cleavage of the C and N terminal peptides involves triple helix dependent metalloproteinases, namely procollagen C and N-proteinase, which cleave at specific sites to yield tropocollagen. The tropocollagens are then able to spontaneously aggregate into quarter-staggered fibrils which is directed by the presence of clusters of hydrophobic and charged amino acids on the surface of the molecules (refer to Figure 1.8) (Rossert and de Crombrugghe 1996).

1.9.3 Collagen Cross-Linking

Once the fibrils are formed, the collagen molecule can be further stabilised by the formation of intrafibrillar cross-links (form within the same triple helix) and interfibrillar cross-links (from between two different triple helices). It is partly due to these cross-links that connective tissues have such a high mechanical strength. Even in bone where mineral is often incorrectly thought to be the main provider of tensile strength, the absence or reduction in cross-links can have devastating effects on the integrity of the tissue.

Several types of cross-linking are formed in the collagen matrix, including disulphide bonding, enzymatic and non-enzymatic links. The proportions can vary depending upon the tissue and type of collagen. For example, in collagen types III, IV, VI and IX, covalent cross-links can be formed by disulphide bonds, however, type I does not contain the cysteine residues needed to form disulphide bridges. Hence within collagen type I, cross-links are formed as a result of the oxidation of specific lysine and hydroxylysine residues in the telopeptide regions by the enzyme lysyl oxidase, a copper dependent amine oxidase enzyme, to form either lysine or hydroxylysine aldehyde residues (Bailey and Paul 1997) (it is important to note that this is not exclusive to type I collagen and such enzymatic cross-links can be formed in collagen types III, IV, VI and IX).

Immature Enzymatic Cross-links:

There are two major pathways of lysyl oxidase mediated collagen cross-linking and they are dependent upon whether the residue in the telopeptide is lysine or hydroxylysine.

a) Lysine aldehyde derived cross-links

Lysine aldehydes (allysines) are able to form both intra and intermolecular collagen cross-links. Intramolecular cross-links can be formed when two adjacent aldehydes exist in the N-telopeptide region of the same molecule, which can condense to form an aldol condensation product (ACP) (refer to Figure 1.9). Intermolecular cross-links are formed when lysine aldehydes in the telopeptides react with either lysine or hydroxylysine residues in the helices of adjacent molecules. This occurs in the form of a condensation reaction and forms a Schiff base, which is reducible under certain conditions, i.e. during

sodium borohydride (NaB^3H_4) reduction. The most common lysine aldehyde cross-link involves a helical hydroxylysine. The resultant linkage is the aldimine dehydro-hydroxylysinonorleucine (deH-HLNL), which when reduced with borohydride, becomes hydroxylysinonorleucine (HLNL) (Kielty *et al.*, 1993) (refer to Figure 1.9). The lysine aldehyde pathway predominates in adult skin, cornea and sclera.

b) Hydroxylysine aldehyde derived cross-links

The immature cross-links formed through reactions of a hydroxylysine aldehyde (hydroxyallysine) with helical hydroxylysine or lysine residues follow essentially the same process as for lysine aldehyde derived intermolecular cross-links. However, in this case the Schiff base products, derived from reactions between a hydroxylysine aldehyde and a lysine or hydroxylysine residue on adjacent molecules, are unstable and therefore undergo spontaneous Amadori rearrangement to produce hydroxylysino-5-keto-norleucine (HLKNL), also named hydroxylysino-5-oxonorleucine (HLONL), which is reduced to dihydroxy-lysinonorleucine (diHLNL) (Eyre 1996; Robins 1999) (refer to Figure 1.10). The hydroxylysine aldehyde pathway predominates in ligament, bone, cartilage and most tendons.

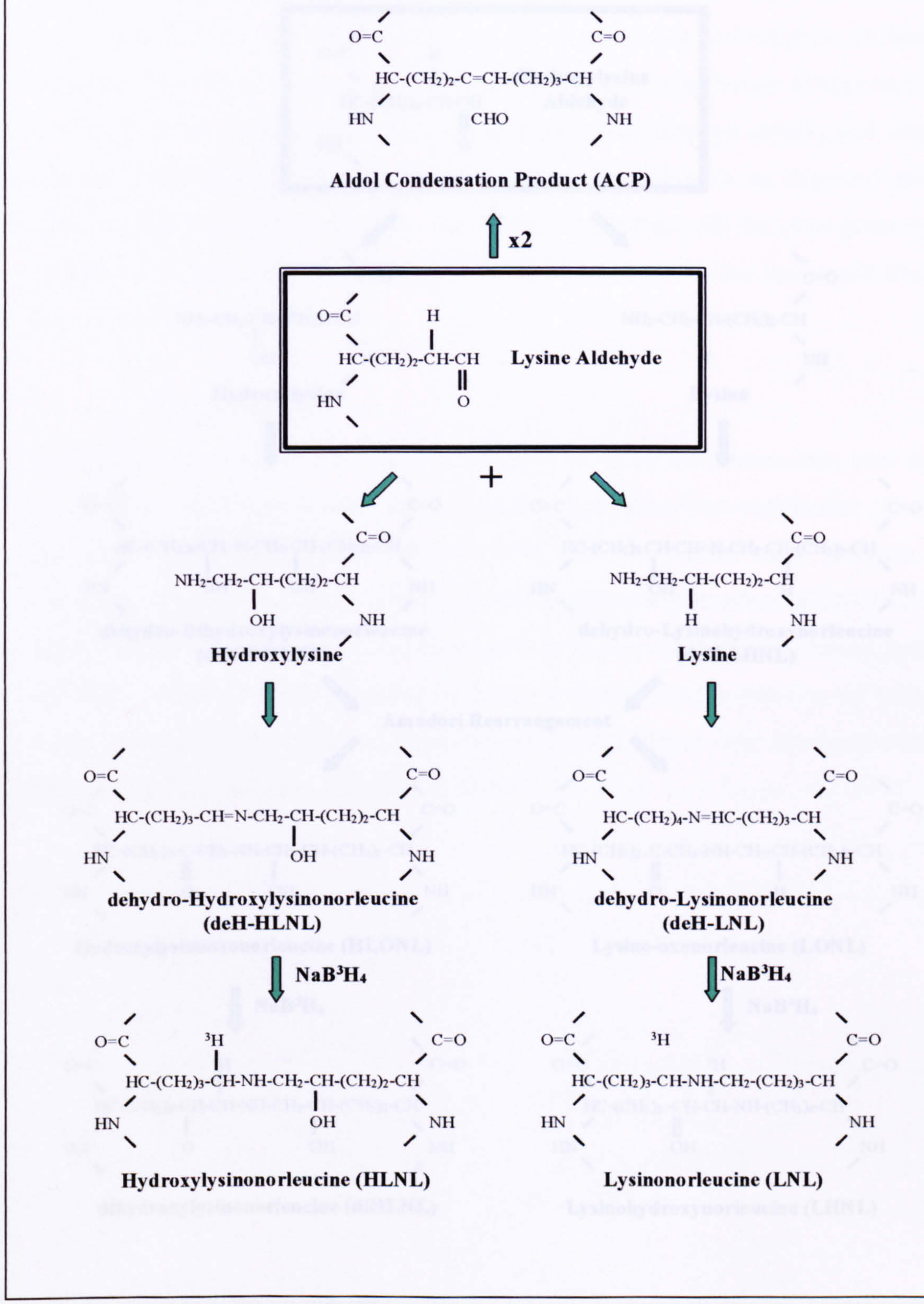


Figure 1.9: Lysine aldehyde immature cross-link pathways.

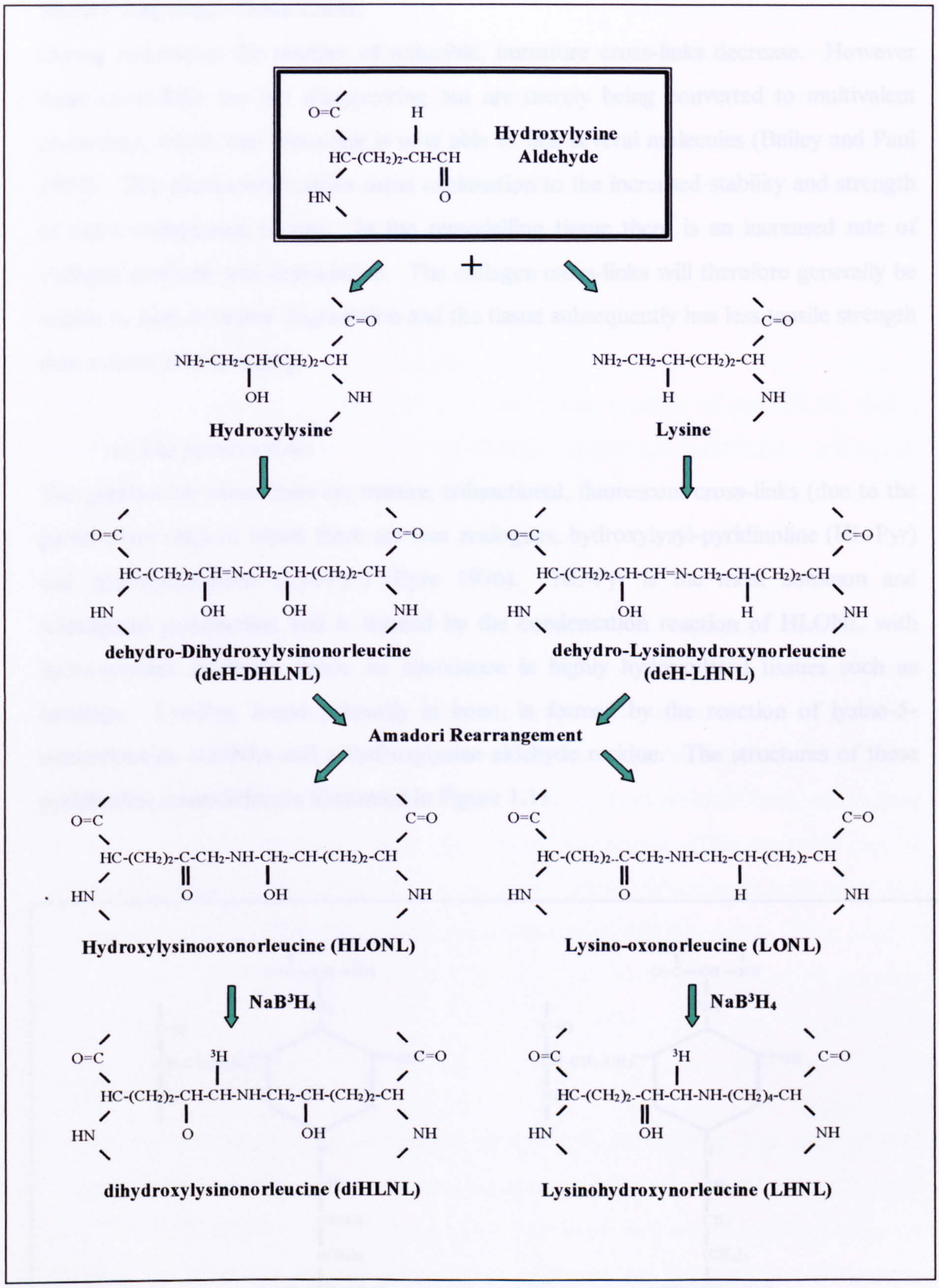


Figure 1.10: Hydroxylysine aldehyde immature cross-link pathways.

Mature Enzymatic Cross-Links:

During maturation the number of reducible, immature cross-links decrease. However these cross-links are not disappearing but are merely being converted to multivalent cross-links, where one cross-link is now able to link several molecules (Bailey and Paul 1997). This phenomenon gives some explanation to the increased stability and strength of older collagenous tissues. In the remodelling tissue there is an increased rate of collagen synthesis and degradation. The collagen cross-links will therefore generally be unable to mature before degradation and the tissue subsequently has less tensile strength than a tissue that is ageing.

a) The pyridinolines

The pyridinoline cross-links are mature, trifunctional, fluorescent cross-links (due to the pyridinoline ring) of which there are two analogues, hydroxylysyl-pyridinoline (HL-Pyr) and lysyl-pyridinoline (Lys-Pyr) (Eyre 1996). HL-Pyr is the most common and widespread pyridinoline and is formed by the condensation reaction of HLONL with hydroxylysine aldehyde, hence its abundance in highly hydroxylated tissues such as cartilage. Lys-Pyr, found primarily in bone, is formed by the reaction of lysino-5-oxonorleucine (LONL) and a hydroxylysine aldehyde residue. The structures of these pyridinoline cross-links are illustrated in Figure 1.11.

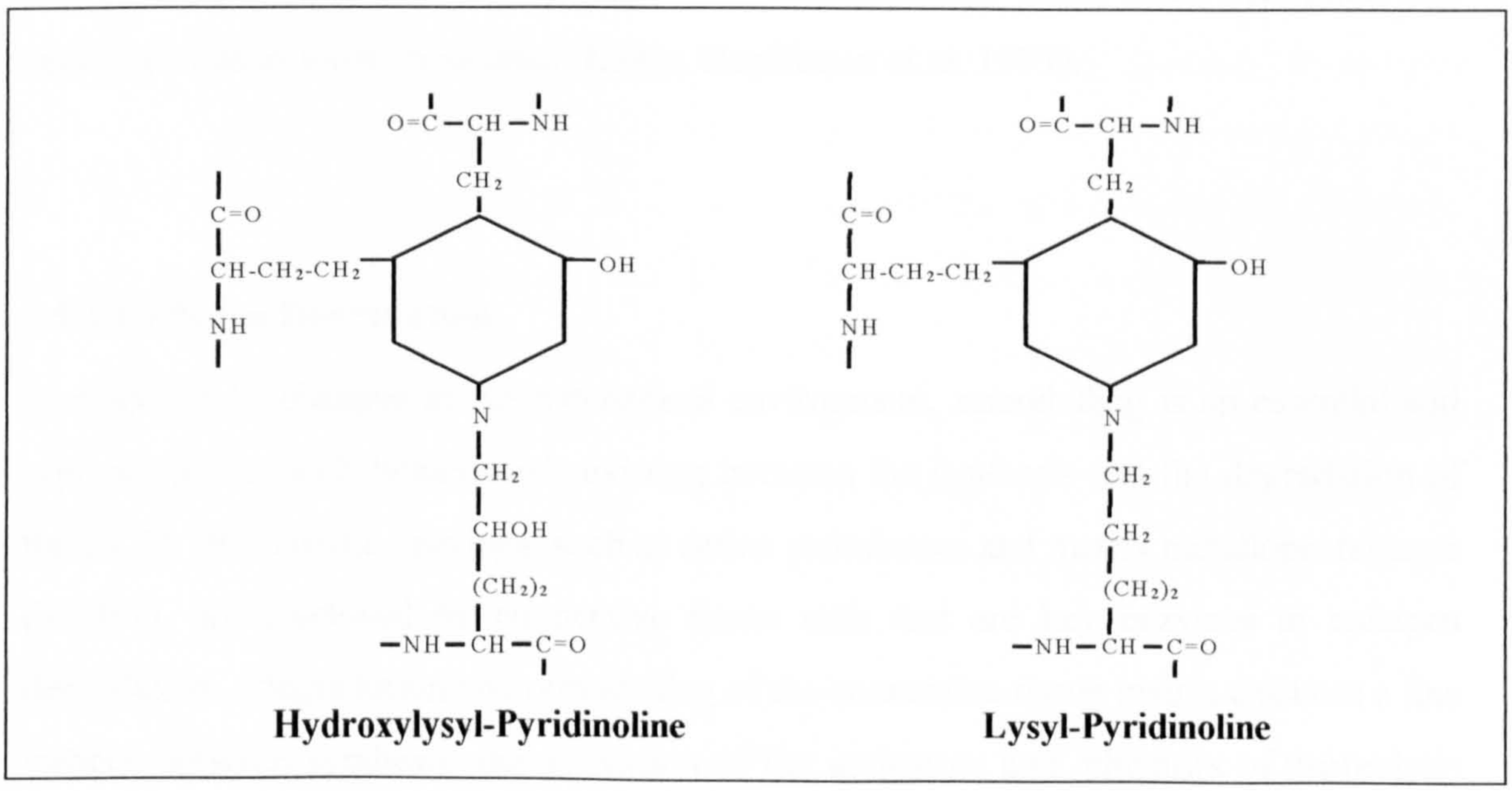


Figure 1.11: Structures of the pyridinoline cross-links.

b) The pyrrole cross-link

The pyrrole cross-links are mature trivalent collagen cross-links and are thought to be formed due to the condensation of a lysyl telopeptide aldehyde and the immature cross-link HLONL (Scott, Qian et al. 1983; Kuypers, Tyler et al. 1992). This formation pathway suggests that if the telopeptide lysine is hydroxylated, pyridinoline would form, if not then the pyrrole is favoured, a theory postulated by Robins *et al.*, (1983) (Robins 1983; Robins and Duncan 1983).

c) Histidinohydroxylysinonorleucine

Histidinohydroxylysinonorleucine (HHL) is the mature product of deH-HLNL and is thought to be due to the condensation of a histidine residue and deH-HLNL. It is mainly found in skin and the cornea and to a lesser extent in ligament.

Non-Enzymatic Cross-links:

With increasing age, or a reduction in the rate of turnover, additional cross-links are formed due to non-enzymic glycosylation (glycation). During this process a sugar group (frequently glucose) reacts nonenzymically with the free amino group of lysine or hydroxylysine residues. This form of glycosylation involves the chemical reaction of a sugar aldehyde or ketone with a free amino group to form a Schiff base, which may undergo a rearrangement to form a fairly stable ketoamine. This structure can be subsequently degraded into a reactive α–dicarbonyl group, which react with other free amino groups to form cross-links (Kielty, Hopkinson et al. 1993).

1.9.4 Collagen Degradation

In response to changes in the mechanical environment, remodelling is an essential and normal process with homeostasis existing between the synthesis and the degradation of the ECM. Proteolytic enzymes, such as serine proteinases and matrix metalloproteinases (MMPs), are produced by connective tissue cells and are key enzymes in collagen degradation. Degradation and remodelling of the connective tissue matrix exists as a fine balance between synthesis, the activation of the activators and inhibitors of proteolytic

enzymes, and their control by external stimuli such as cytokines and growth factors (Mauviel 1993).

Despite the normal homeostatic role of proteolytic enzymes in connective tissue metabolism, these enzymes can, in disease, lead to excessive destruction of the collagen, which can have deleterious effects on the mechanical integrity of the tissue. The MMPs, for example, have been considered for some time to be fundamental in the pathogenesis of equine joint disease, as the breakdown of articular cartilage matrix in humans is thought to occur primarily by enzymatic degradation (Ehrlich, Armstrong et al. 1986).

Several functionally related proteases with diverse substrate specificity coordinate the degradation and resorption of collagen. The main proteinases involved can be classified into four groups according to the amino acid or chemical group at the catalytic domain of the enzyme: the cysteine-, the aspartate- and the serine-dependent proteases, and the metalloproteinases. Moreover, there are two major proteolytic pathways: 1) the intracellular pathways in which proteins are hydrolysed by cysteine- and aspartate-proteases at low pH and, 2) the extracellular pathways, in which pathways are enzymatically digested by serine-proteases and metalloproteases that generally act at neutral pH. However, some enzymes that are active extracellularly, cathepsins B and D, have higher specific activity at low pH (Martel-Pelletier, Welsch et al. 2001).

The collagen molecule is very resistant to proteolytic attack by nature of its integral structure. The intra- and intermolecular cross-links, which confer helical stability, render it a poor target for proteolytic enzymes. The process of the molecular lysis is thought to be initiated extracellulary, via the action of either the metallo-, serine, cysteine and/or aspartate proteinases, cleaving the collagen at either the telopeptide or the helical region depending upon the proteinase involved. The cleaved products are then susceptible to extracellular degradation by other proteolytic enzymes, or are phagocytosed by surrounding cells and are presented for intracellular digestion by enzymes such as the cysteine proteinases (refer to Figure 1.12).

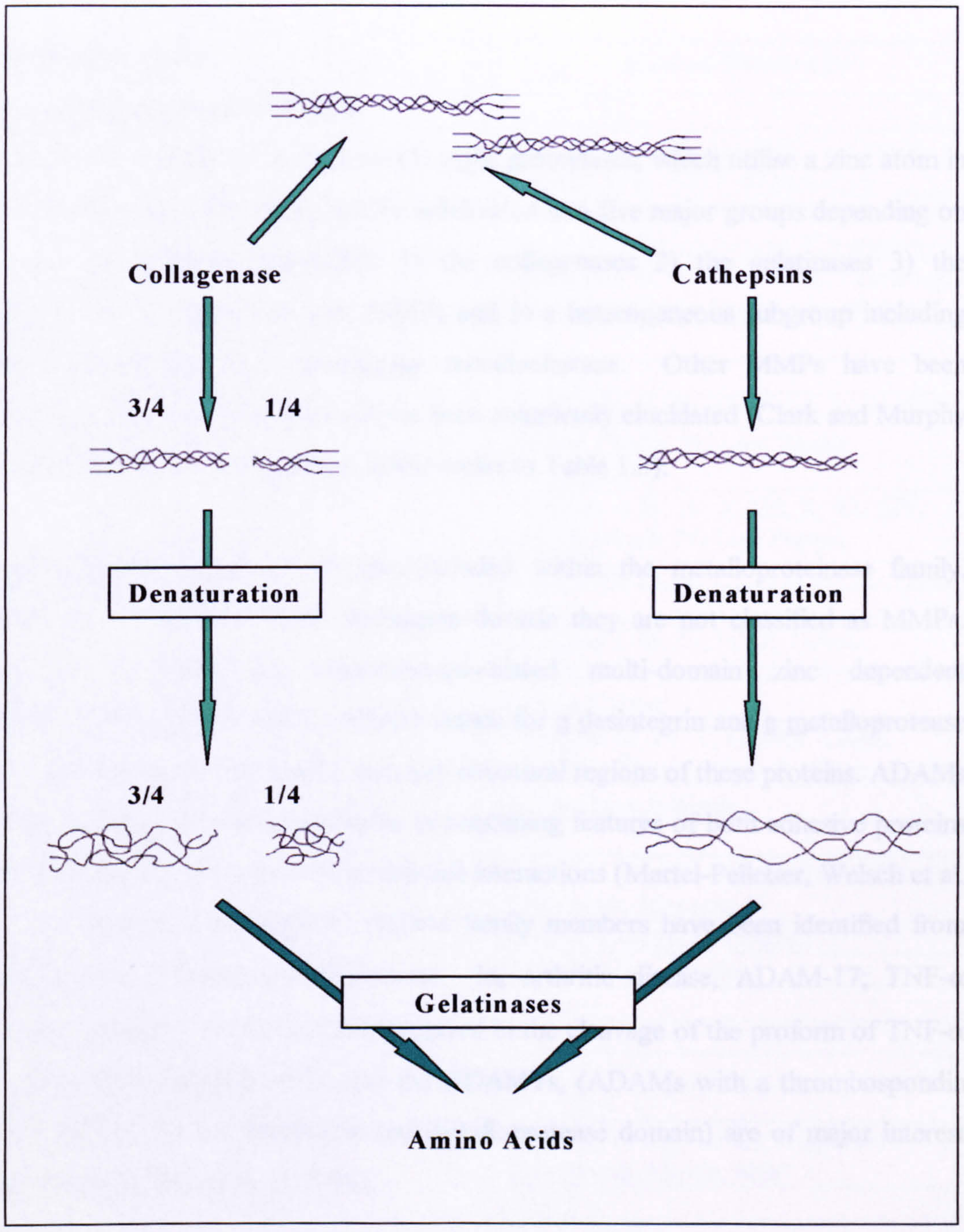


Figure 1.12: Enzymatic degradation of collagen molecules by collagenase (e.g. MMPs-1, 8, 13 and 18) and cathepsins (e.g. cathepsins D, an aspartic proteinase). Collagenase cleaves the molecule at a specific site ¾ along the triple helix. Cathepsins cleave the molecule at the telopeptide releasing the molecule from the intact fibre. Cleaved fragments then denature and are digested by other proteinases, such as the gelatinases (MMP-2 and -9). Adapted from Bailey *et al.*, (1997).

1) Metalloproteinases:

Matrix Metalloproteinases (MMPs)

The MMPs are a family of at least twenty eight proteinases, which utilise a zinc atom in their catalytic mechanism. They can be subdivided into five major groups depending on their size and substrate specificity: 1) the collagenases 2) the gelatinases 3) the stromelysins 4) the membrane-type MMPs and 5) a heterogeneous subgroup including matrilysin, enamelysin and macrophage metalloelastase. Other MMPs have been identified but their functions have not yet been completely elucidated (Clark and Murphy 1999; Martel-Pelletier, Welsch et al. 2001) (refer to Table 1.3).

The *adamalysins* (ADAMs) are also included within the metalloproteinase family, although due to their distinctive disintegrin domain they are not classified as MMPs. These are a family of membrane-associated multi-domain zinc dependent metalloproteases (Blobel 1997). ADAM stands for a desintegrin and a metalloprotease domain, and their name reflects the two key structural regions of these proteins. ADAMs are unique among cell–surface proteins in containing features of both adhesive proteins and proteases and in having a role in cell-cell interactions (Martel-Pelletier, Welsch et al. 2001). At present, more than 25 ADAM family members have been identified from mammalian and non-mammalian sources. In, arthritic disease, ADAM-17; TNF-α converting enzyme (TACE), which is involved in the cleavage of the proform of TNF-α to its active form (Blobel 1997), and the ADAMTs, (ADAMs with a thrombospondin motif, in addition to the disintegrin and metalloprotease domain) are of major interest (Martel-Pelletier, Welsch et al. 2001).

MMP Number	Enzyme	Known Substrates
Collagenases:		
MMP-1	Interstitial Collagenase-1	Collagens I, II, III, VII, VIII, X, Gelatin, Aggrecan, PG Link Protein, Casein
MMP-8	Neutrophil Collagenase-2	Collagens I, II, III, V, VII, VIII, X, Gelatin, Aggrecan, PG Link Protein, Fibronectin
MMP-13	Collagenase-3	Collagens I, II, III, IV, Gelatin, Plasminogen Activator Inhibitor 2, Aggrecan, Perlecan
MMP-18	*Xenpus* Collagenase-4	?
Gelatinases:		
MMP-2	Gelatinase A	Collagens I, II, III, IV, V, VII, X, XI, XIV, Gelatin, Elastin, PG Link Protein, Fibronectin
MMP-9	Gelatinase B	Collagens I, III, IV, V, VII, X, XI, XIV, Gelatin, Elastin PG Link Protein, Fibronectin
Stromelysins:		
MMP-3	Stromelysin-1	Collagens I, III, IV, IX, X, Gelatin, Aggrecan, Versican, PG Link Protein, Elastin, Casein
MMP-10	Stromelysin-2	Collagens III, IV, V, Gelatin, Casein, Aggrecan, Elastin, PG Link Protein
MMP-11	Stromelysin-3	α_1-proteinase Inhibitor
Membrane-type MMPs (MT-MMP):		
MMP-14	MT-MMP-1	Collagens I, II, III, Gelatin, Casein, Elastin, Fibronectin, Aggrecan
MMP-15	MT-MMP-2	MMP-2, Gelatin, Fibronectin
MMP-16	MT-MMP-3	Pro-MMP-2 (activation)
MMP-17	MT-MMP-4	?
MMP-24	MT-MMP-5	?
MMP-25	MT-MMP-6	?
Heterogeneous subgroup:		
MMP-7	Matrilysin	Collagens IV, X, Gelatin, Aggrecan, PG Link Protein, Elastin, MBP
MMP-12	Metalloelastase	Elastin
MMP-19	RASI-1	?
MMP-20	Enamelysin	Enamel matrix
Other MMPs:		
MMP-4	Identified as MMP-3	?
MMP-5	Identified as MMP-2	?
MMP-6	Identified as MMP-3	?
MMP-21	X-MMP	?
MMP-22	C-MMP	?
MMP-23	CA-MMP or MIFR-1	?
MMP-26	Endometase	?
MMP-27	Epilysin	?
MMP-28	Newly identified	?

Table 1.3: Matrix metalloproteinases of the extracellular matrix. Adapted from Clark *et al.*, (1999) and Martel-Pelletier *et al.*, (2001).

Molecular Structure of MMPs

The MMPs, although the product of different genes, share structural and functional properties, including optimal activity at neutral pH and the requirement of calcium and zinc for biological activity. MMPs have a common domain structure with a signal peptide, a propeptide, a catalytic domain, a hinge region and a haemopexin-like C-terminal domain, although individual MMPs have variations on this general structure (Clark and Murphy 1999):

Signal peptide - This directs the translational product to the ER where it can be cleaved prior to being secreted from the cell.

Propeptide - The function of this region is to maintain the proenzyme in its latent state prior to activation by external stimuli. This function is provided by the cysteine-switch mechanism, which involves the co-ordination of Cys^{73} to the active centre zinc atom.

Catalytic domain (162-173 amino acids) – This contains a zinc binding region, HExxHxxGxxH, in which three histidines are likely to be zinc binding ligands. This domain requires calcium ions for stability and also activation.

Hinge region – This region attaches the catalytic domain to the haemopexin-like domain, and influences substrate specificity.

C-terminal haemopexin-like domain (202-213 amino acids) – Haemopexin is a blood glycoprotein with a high affinity for haem. This domain is essential for the collagenases to cleave the triple helix, but is not essential for all enzyme activity. This domain may also bind TIMP- 2 and-1, the tissue inhibitors of MMPs, to the gelatinases A and B respectively. This domain is found in all MMPs except MMP-7.

The MT-MMPs (MMP 14-17, 24-25) also have a transmembrane domain and a cytoplasmic tail at the C-terminal end, and in common with MMP-11 have a sequence containing a potential furin cleavage site at the N-terminal end of the catalytic domain (which permits intracellular activation). The gelatinases (MMP-2 and –9) additionally

have an insert of three gelatin binding fibronectin type II repeats in the catalytic domain, with MMP-9 having a collagen-like sequence C terminal to the catalytic domain (Clark and Murphy 1999).

Regulation of MMPs

The synthesis, activation, and inhibition of MMPs are tightly regulated at several levels in order to maintain an appropriate balance between the synthesis and degradation of connective tissues. The lack of regulation in diseased tissue can lead to excessive activity and resulting degradation by these enzymes. Regulatory pathways are found at both the transcriptional level, where cytokines such as IL-1β and TNF-α and some growth factors including epidermal growth factor (EGF), platelet derived growth factor (PDGF) and TGF-β can stimulate MMP gene expression, and the post-transcriptional level, where plasmin or other active MMPs activate the secreted latent MMPs, which in turn are inhibited by specific tissue inhibitors (TIMPs). These TIMPs are co-ordinately expressed with the MMPs (Martel-Pelletier, Welsch et al. 2001).

Activation of MMPs

In vivo, most MMPs, except MT-MMPs and MMP-11, are secreted as latent proenzymes and are subsequently activated extracellularly. Collagenase is normally activated by stromelysin, but plasmin (produced from plasminogen by the action of tissue or urokinase-type plasminogen activators), kallikerin and cathepsin B can also activate collagenase, and possibly other MMPs. Plasmin and other proteinases that activate collagenase activate stromelysin. Evidence suggests that latency is attributable to the formation of an intramolecular complex between a single cysteine residue in the propeptide domain and the essential zinc atom in the catalytic domain (these being the only two domains common to all MMPs). Activation is associated with detachment of the cysteine residue from the complex and is referred to as the 'cysteine-switch' mechanism of activation (Stamenkovic 2003).

Inhibition of Active MMPs

MMP activity is tightly controlled by several endogenous inhibitors. These include α_2-macroglobulin and the tissue inhibitors of metalloproteinases (TIMPs).

i) α_2-macroglobulin

α_2-macroglobulin is able to bind to all proteinases and creates a complex that is itself bound and irreversibly cleared by scavenger receptors (Stamenkovic 2003). Although, being the most prominent and widespread inhibitor of all proteases, because of its size, α_2-macroglobulin is mainly functional in serum and other bodily fluids, so it is the action of the TIMPs in connective tissue that remains the most significant.

ii) Tissue Inhibitors of Metalloproteinases (TIMPs)

TIMPs, produced by the same cells that secrete MMPs, can irreversibly inhibit the action of MMPs. To date four TIMPs have been described (Clark and Murphy 1999):

- TIMP-1, a 28 kDa glycosylated protein
- TIMP-2, a 22 kDa nonglycosylated protein
- TIMP-3, a 21-27 kDa protein that binds to the extracellular matrix
- TIMP-4, a 22 kDa protein

TIMPs are highly stable and resistant to heat, due to the presence of six disulphide bonds within their structure. These disulphide bonds hold two domains in rigid conformation. One domain is thought to be responsible for inhibition and the other enzyme recognition (Stamenkovic 2003). TIMPs inhibit MMPs by forming a 1:1 enzyme-inhibitor complex, and are controlled by local cytokine, growth factor and hormone levels. TIMP 1 can bind non-covalently to MMP-9 and is produced by many cells including neutrophils, and can therefore hinder the functions of MMP-9, which is also a major product of neutrophils. TIMP-2 can form non-inhibited complexes with MMP-2, and is important in the cell surface localisation, activation and stabilisation of this enzyme (Yoshizaki, Sato et al. 2002). TIMP-3 has been found expressed in articular cartilage as a strong inhibitor of ADAMs, aggrecanase 1 and aggrecanase 2 (Kashiwagi, Tortorella et al. 2001). TIMP-4 has been found in cancer cells and appears responsible for the cell surface activation of proMMP-2 (Martel-Pelletier, Welsch et al. 2001).

The MMPs Involved in the Degradation of the Fibrillar Collagens (I, II and III)

Those MMPs that are thought to be largely involved in the degradation of bone and ligament collagen will be discussed in more detail.

a) The Collagenases

The triple helical domain of the fibrillar collagens is highly resistant to proteolytic cleavage. The collagenases, however, are able to cleave all three collagen molecules at a specific loci within the triple helical domain to yield ¾ and ¼ fragments. The main members of this family are collagenase-1 (MMP-1), collagenase-2 (MMP-8) and collagenase-3 (MMP-13).

Collagenase-1 or MMP-1 is secreted as a 52kDa zymogen, with a minor 57kDa glycosylated form, being converted to the active forms of 42 and 47kDa respectively (Clark and Murphy 1999). Collagenase-1 has many potential substrates as listed in Table 1.3, however, its most important targets are the fibrillar collagens, although it has greater specificity for type III then type I and has poor activity against type II collagen. Collagenase-1 is expressed in most connective tissues. Collagenase-2 or MMP-8 is produced specifically by neutrophils and is a distinct gene product from collagenase-1 (Murphy and Docherty 1992). The main substrates, as for Collagenase-1, are the fibrillar collagens, although this MMP shows a greater activity towards type I collagen than type III, with type II again being a weak substrate. Collagenase-3 or MMP-13, which is homologous to rodent Collagenase-1, was first discovered in breast carcinoma (Wernicke, Seyfert et al. 1996). Its principal substrates are again the fibrillar collagens, primarily collagen type II, hence its activity is important in cartilage degradation.

b) The Gelatinases

The gelatinases, MMP-2 and MMP-9, both specifically bind and degrade all the denatured collagens, gelatin, and are also capable of cleaving native type IV collagen. The two gelatinases have similar substrate specificity, gene exon structure and chromosomal location, however their control at transcriptional level is markedly different due to profound differences in their promoter elements (Murphy 1995). The binding of these enzymes to gelatin is facilitated via the insertion of fibronectin -like repeats into the catalytic domain (Murphy and Docherty 1992).

MMP-2 or Gelatinase A is the most widespread of all the MMPs, being produced constitutively by all connective tissue cells (Murphy 1995). It is thought to be responsible for the turnover and maintenance of the integrity of healthy ECM components by the remodelling or removal of damaged collagen molecules. MMP-9 or Gelatinase B expression is induced by cytokines and growth factors and is mainly associated with inflammatory processes and particularly neutrophils (Tarlton, Vickery et al. 1997).

c) The Stromelysins

The stromelysin group of MMPs is comprised of three members: Stromelysin-1, -2 and –3. Stromelysin-1 or MMP-3, has a broad substrate specificity including collagen types III, IV, IX, X and gelatin, and types I, II and XI in the telopeptide regions. Non-collagenous substrates include proteoglycans, fibronectin, laminin and the serpins. A relationship has been demonstrated between the level of stromelysin–1 and the severity of proteoglycan degradation and the enzyme has also been implicated in the enzymatic cascade responsible for the activation of MMP-1 (Martel-Pelletier, Welsch et al. 2001). Stromelysin-2 or MMP-10 has a 78% sequence homology with MMP-3; its activation is also similar to that of MMP-10, except that neutrophil elastase is ineffective in this process. The enzyme has been detected with Stromelysin-3 or MMP-11 in the synovial fibroblasts of rheumatoid arthritis (RA) patients (Martel-Pelletier, Welsch et al. 2001).

d) Membrane-type MMPs (MT-MMPs)

The MT-MMPs are a group of six membrane anchored MMPs. Several studies have suggested a role for MT-MMPs in the degradation of the ECM. MT1-MMP is expressed in human articular cartilage and possesses collagenolytic activity and MT3-MMP is intensely expressed in OA and RA synovium (Pap, van der Laan et al. 2000). MT-MMP1, MT2-MMP, MT5-MMP and MT6-MMP are known to activate proMMP-2 and MT1-MMP and MT2-MMP activate proMMP-13 (Collagenase-3) (Knauper, Will et al. 1996).

2) Serine Proteinases

The serine proteinases are the largest family of endopeptidases. They are involved in the extracellular pathway of ECM degradation, being primarily an activator of the MMPs.

They are mostly active at a neutral pH, and require a serine, histidine and aspartic acid residue to hydrolyse the peptide bond. They are released from inflammatory cells during the tissue resorption and degradation of inflammation (Murphy and Docherty 1992).

The most important serine proteinases are the plasminogen/plasminogen activator (PA) system. Plasminogen (90kDa), an abundant serum protein, is cleaved by the PAs and converted to the broad specificity proteinase, plasmin. Both plasmin and plasminogen can bind to ECM components and are thereby protected by its inhibitors. However, the production of plasmin is controlled by the rate-determining PAs. Plasmin has good activity against fibrin, as well as fibronectin, laminin, proteoglycans, and type IV collagen, and is involved in the activation of many MMPs (Murphy, Frank et al. 1993). There are two distinct PAs with differing functions: Tissue-type PA (tPA) and Urokinase-type PA (uPA). tPA (70kDa) is involved in regulating the clotting process and uPA(50kDa) is involved in fibrinolysis as well as biological remodelling (Murphy and Docherty 1992).

Some of the other serine proteinases are cathepsin G and neutrophil elastase. Cathepsin G is a serine proteinase that is found as a proenzyme in the azurophilic granules of neutrophils and to a lesser extent, macrophages and monocytes. It is active against degraded collagen products and also proteoglycans, fibronectin, and laminin. The primary site of activity is intracellularly in the lysosomes, but it can also function extracellularly. Neutrophil elastase is a single chain glycoprotein stored in the azurophilic granules of neutrophils. It has a broad specificity and can act on a number of host proteins outside the neutrophil. It can cleave among others, fibrillar type III collagen, native elastin, proteoglycan core protein, vitronectin and laminin. Its role is mainly involved in inflammation (Murphy, Frank et al. 1993).

The inhibitors of the serine proteinases are the serpins, the main members being α_1-proteinase inhibitor, α_2-antiplasmin, and plasminogen activator inhibitors (PAIs).

3) Cysteine Proteinases

Cysteine proteinases are widely distributed amongst living organisms and can be subdivided into 14-20 different families, the main ones being calpain and papain families.

Four of the members of the papain family are ubiquitous in the lysosomes of animals: cathepsins B, C, H and L, with cathepsin S having a more restricted localisation (Turk, Turk et al. 1997). They are synthesised in the lysosomes as proenzymes and have optimal activity at an acidic pH, once activated. They are mainly involved in the intracellular digestion of phagocytosed matrix fragments. The inhibition of the cysteine proteinases is via the cystatins, of which there are three groups.

4) Aspartic Proteinases

All of the proteinases of this family require an aspartic residue as part of their catalytic site and function optimally, as with the cysteine proteinases, at an acidic pH. The main member of this group, cathepsin D, is synthesised as a proenzyme and then packaged into cellular lysosomes. The proenzyme is then slowly cleaved into the active form, and is involved in the intracellular digestion of phagocytosed ECM material (Clark and Murphy 1999).

1.10 HYPOTHESIS AND AIMS

The effects of high-intensity exercise and age on the midcarpal joint of the racing TB has to date received scant attention, especially with the regard to the effect of exercise and age on the ECM metabolism of the radial (Cr) and third (C3) carpal bones, and the biomechanics and ECM metabolism of the medial palmar intercarpal ligament (MPICL).

The hypotheses of this thesis are:

1. During exercise-induced remodelling in the Cr and C3 of racehorses, an increase in bone formation results in subchondral cortical bone thickening and hence increased bone density.
2. The organic matrix that is being formed during this remodelling process in both the subchondral cortical bone and the trabecular bone has inferior qualities, which may result in weaker bone, predisposing to fracture.
3. An altered bone composition would alter the loading on the articular cartilage and contribute to cartilage pathology, specifically articular cartilage degeneration.
4. High-intensity exercise, as experienced by the racing TB, causes increased tearing of the MPICL, specifically the dorsolateral (DL) and dorsomedial (DM) branches, which may be either mediated or contributing to increased ECM metabolism. This increased prevalence of tearing would decrease the stability of the midcarpal joint and hence contribute to osteochondral damage.

This thesis therefore aims to:

- Determine the effects of high-intensity exercise on the inorganic properties and the ECM metabolism of the Cr and C3 by studying horses that have been subjected to racing and race-training and from horses that have not raced.
- Determine the effects of racing and race-training on the biomechanics and molecular composition of the DL and DM branches of the MPICL.
- Determine if there is any relationship with the degree of cartilage pathology and the inorganic and organic properties of the Cr and C3.
- Determine if there is any relationship with the degree of cartilage pathology and the degree of ligament tearing.
- Determine any relationships with age and the various parameters quantified.

CHAPTER TWO

The Effect of Exercise and Relationship of Age on the Mineral Component of the Midcarpal Joint of the TB Horse

2.1 INTRODUCTION

As outlined in Chapter One, exercise can alter bone mass and strength, causing substantial changes in the mechanical properties and density of the bone. Increased bone density (sclerosis/ loss of the normal trabecular pattern) is commonly identified in diseased bone of the Cr and C3 of horses (O'Brien, DeHaan et al. 1985). In one report, fracture of the C3 was accompanied by radiographic evidence of sclerosis in 90% of the horses evaluated (De Haan, O'Brien et al. 1987). However, a degree of exercise-induced sclerosis is probably a normal adaptive modelling and remodelling response to increased mechanical demand (Butler, Colles et al. 1993). Firth *et al.*, (1999) demonstrated that the subchondral bone density, quantified using dual energy x-ray absorptiometry (DEXA), in the dorsal regions of the Cr and C3 was greater in treadmill exercised horses compared to non-treadmill exercised controls, illustrating the localised responsiveness of bone tissue to the stresses of an exercise regime (Firth, Delahunt et al. 1999). Additionally, in a study by Young *et al.*, (1991) the stiffness of bone and area fraction (a quantitative measure of bone density) of the C3 of treadmill exercised horses increased in response to exercise. They also found a strong correlation between stiffness, radiographic density and area fraction, with a large gradient in subchondral bone stiffness being identified at the dorsomedial margin of the radial facet of the C3, thus indicating that subchondral bone responds to the stress of exercise by increasing bone formation in an attempt to increase strength (Young, Richardson et al. 1991).

The effects of exercise on bone density have also been examined in the racing Greyhound. Several studies have examined site-specific remodelling of distal limb bones such as the metacarpal bone V (MCB V), (Emmerson, Lawes et al. 2000; Johnson, Skinner et al. 2001; Lipscomb, Lawes et al. 2001) and the central tarsal bone (CTB) (Muir, Johnson et al. 1999; Johnson, Muir et al. 2000) using DEXA, CT and

histomorphometry. Racing Greyhounds running on anti-clockwise tracks were found to have increased cortical thickness and BMD, in the left MCB V, and the right CTB compared to the contralateral limb, and compared to non-raced controls (Johnson, Muir et al. 2000).

To date, limited information exists on the relationship of age and bone density in the horse. Studies have examined the adaptive response of equine bone to exercise with age, but these have primarily been conducted on young foals (Barneveld and van Weeren 1999; Cornelissen, van Weeren et al. 1999; Firth, van Weeren et al. 1999). The results from these studies suggest that from the age of 5 to 11 months, regardless of exercise intensity, the mean BMD increases. Additionally, Young *et al.*, (1991) found a significant increase in the mean stiffness and area fraction of the C3 with age (Young, Richardson et al. 1991), although again this study was undertaken on young horses aged 2 to 3 years.

Along with the quantification of bone density by DEXA, histomorphometrical analysis can be used to evaluate the rate and distribution of bone remodelling and modelling in response to physical exercise. Previous studies have shown an increase in the subchondral bone area, thickness and osteoid perimeter in the carpus (Murray, Vedi et al. 2001) and the metacarpus (McCarthy and Jeffcott 1992) of the horse with exercise. Similar findings have also been documented in the right CTB of racing Greyhounds (Johnson, Muir et al. 2000). However, to date the influence of age on these parameters has received scant attention.

Since some 70% of the compressive strength of the skeleton is due to its mineral content (Ott, Parfitt et al. 1993), quantification of the mineral content in the Cr and C3 from raced and non-raced horses could provide valuable information on the material properties of the bone. Training has been previously shown to have an effect on the calcium content in the bones of the femoropatellar and MCP joints of horses (Brama, Bank et al. 2001; Brama, TeKoppele et al. 2002; van de Lest, Brama et al. 2002; van de Lest, Brama et al. 2003), being greatest in those subjected to exercise.

Many of the studies to date examining the response of the bone to exercise on the distal limbs of horses have been undertaken on specimens obtained from horses which have been subjected to a treadmill exercise regime, which may consequently not truly reflect the effect of racing and race-training on the skeleton. Therefore, within this chapter the response of the mineral component of the Cr and C3 to exercise has been evaluated on material from TB horses that have been subjected to racing and race-training directly prior to euthanasia for reasons unrelated to carpal pathology and on TB horses that have not raced. The relationship of these parameters with age was also evaluated in this study. Bone quantity has been assessed by obtaining measurements on the BMD, histomorphometry and the total calcium (Ca) and inorganic phosphate (Pi).

2.2 MATERIALS AND METHODS

Details of the materials and solutions used in the following methods are listed in Appendices Four and Five respectively.

2.2.1 Bone Mineral Density (BMD) Quantification

2.2.1.1 Equine Samples

Midcarpal joints from TB racehorses were obtained from the University of Liverpool (UoL) after euthanasia following a racetrack injury (excluding carpal injury). Midcarpal joints from TB non-racehorses were obtained at the UoL or University of Bristol (UoB) veterinary hospitals. Euthanasia was for reasons unrelated to orthopaedic injury or disease.

The racehorses were being actively race-trained and raced up to the time of euthanasia, the non-racehorses had not been in race-training or raced but had undertaken exercise varying from light (walking/ trotting) to medium (cross-country cantering/ galloping) intensity.

For details of the equine samples used within this thesis refer to Appendix Two.

Age and Gender

Table 2.1 shows the age and gender of the horses used in this part of the study.

Raced	Age	Gender	Non-raced	Age	Gender
R1	7	G	**7**	10	M
R2	7	M	**8**	11	M
R3	8	G	**9**	8	G
R4	4	C	**10**	NA	G
R5	6	C	**11**	3	G
R6	7	G	**12**	6	G
R7	7	C	**13**	11	M
R8	7	C	**14**	17	G
R9	6	C	**15**	7	G
R10	6	C	**16**	17	G
R11	7	G	**17**	14	G
R12	4	G			
R13	4	C			
R14	4	F			

Table 2.1: The age (years) and gender of the horses. (C= Colt, F= Filly G= Gelding (neutered male), M=mare, NA= Not Known).

2.2.1.2 Cartilage Pathology Grading

The right and left carpus were opened immediately after euthanasia to expose the midcarpal joint. The degree of articular cartilage pathology of the distal surface of the Cr and the proximal surface of the C3 were graded using a standardised ordinal scoring system (Fuller, Barr et al. 2001) (refer to Table 2.2). This study aimed to assess the response of bone to exercise hence the degree of cartilage pathology was not considered in this part of the study and all samples regardless of cartilage pathology were included. However, the results of the articular cartilage damage assessment will be discussed in detail in Chapter 5. The midcarpal joint was subsequently frozen at –20°C until required.

Score	Articular Cartilage Damage
0	Normal
1	Minimal articular cartilage damage
2	Articular cartilage damage affecting up to 30% of the articular surface of the bone
3	Loss of up to 50% of the articular cartilage from the affected carpal bone
4	Severe loss of cartilage affecting more than 50% of the articular surface

Table 2.2: Scoring system used for the assessment of the degree of cartilage damage (Fuller, Barr et al. 2001).

2.2.1.3 Tissue Sample Site

Bone samples from the Cr and C3 used for all subsequent analyses were taken from sites documented as most prone to pathology (O'Brien, DeHaan et al. 1985; De Haan, O'Brien et al. 1987; Firth, Delahunt et al. 1999; Firth, Goodship et al. 1999). Samples from the Cr were taken ½cm in along the lateromedial (x) axis and 5% along the dorso-palmar (y) axis from the dorsal aspect (refer to Figure 2.1). From this point a $1cm^2$ section was taken providing a piece of bone $1cm^2$ and approximately 4cm in depth.

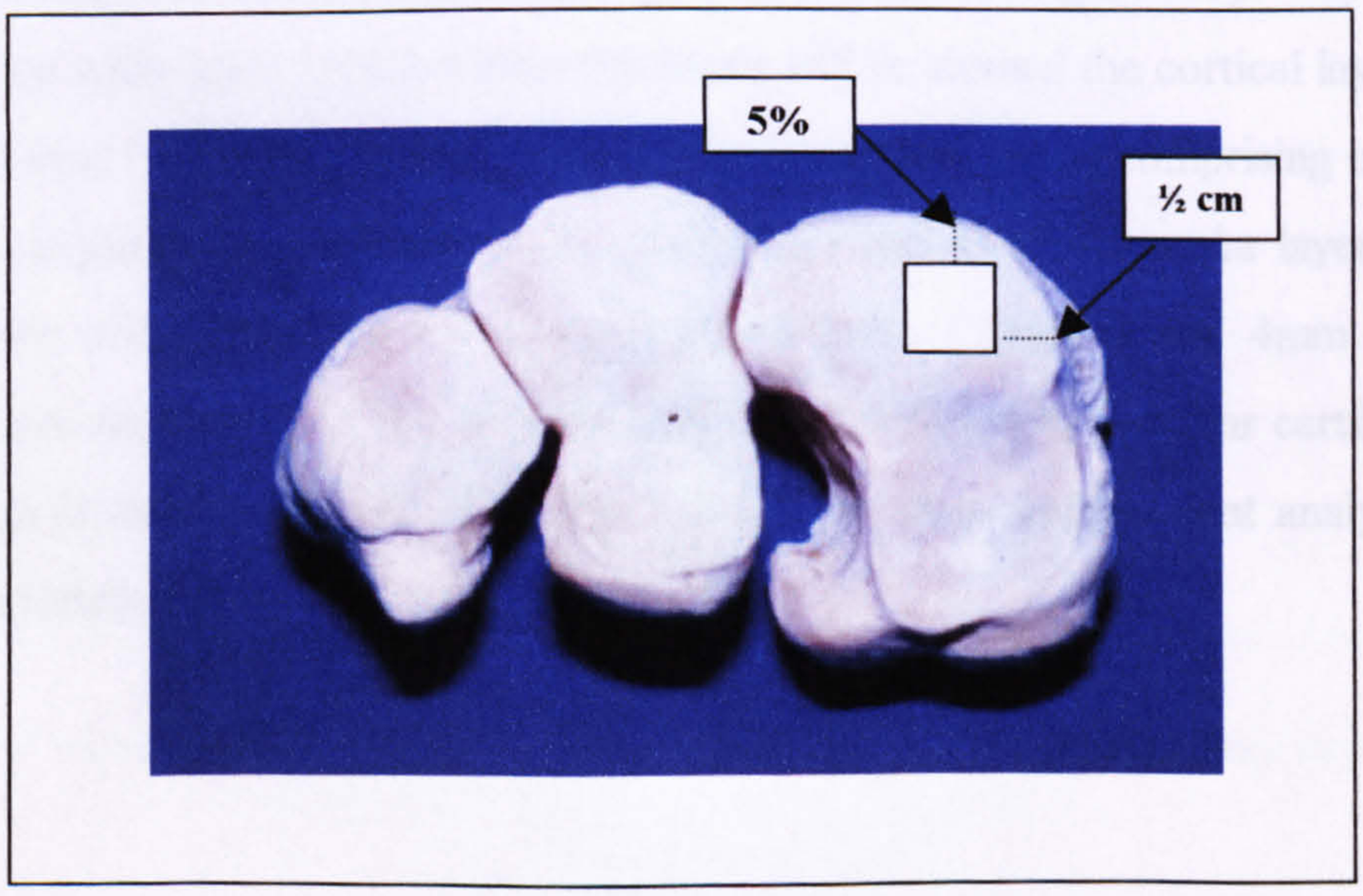


Figure 2.1: Sampling site of the Cr (distal surface).

Bone samples of the C3 were taken 15% along the dorso-palmar (y) axis from the dorsal aspect and mid-way along the lateromedial (x) axis of the radial facet (refer to Figure 2.2). From this point a 1cm^2 section was taken providing a piece of bone 1cm^2 and approximately 3cm in depth.

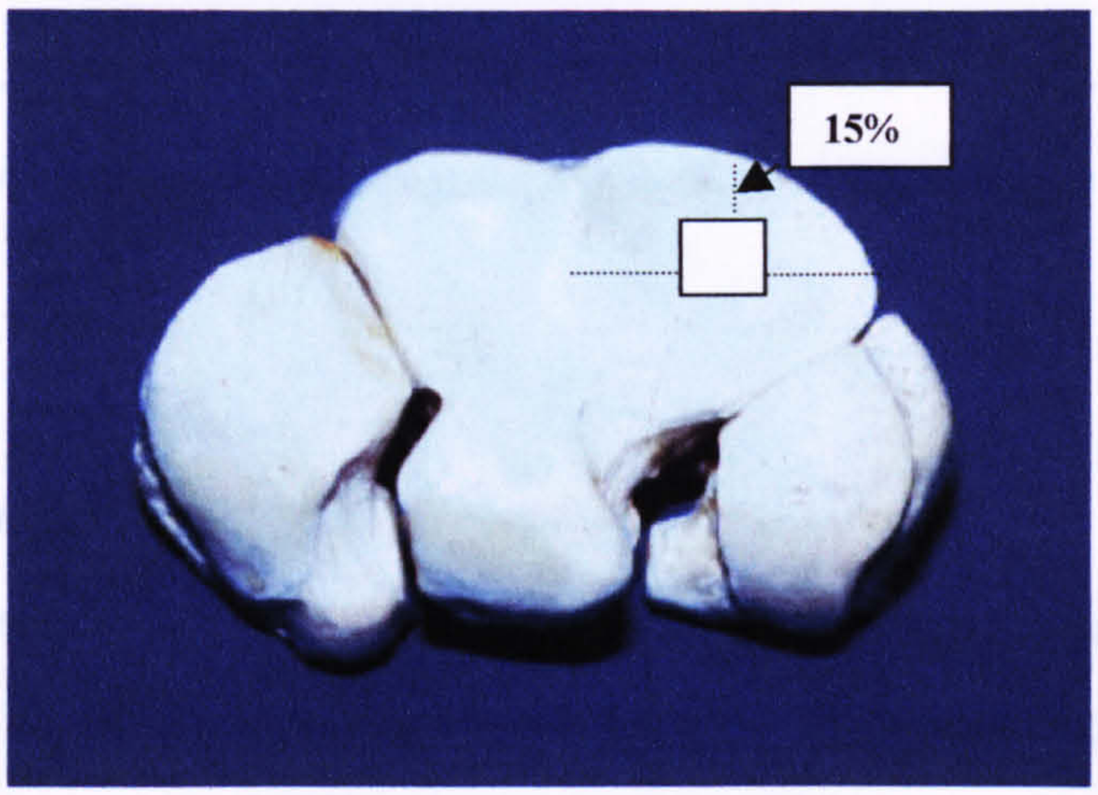


Figure 2.2: Sampling site of the C3 (proximal surface).

The articular cartilage was removed and stored at -20°C. From the blocks of bone, four sampling zones were established, termed layers a-d (refer to Figure 2.3). Layer a is the cortical bone plate layer, which within this thesis will be termed the cortical layer. Layer b is the cortical bone plate/trabecular layer (the 'transition' layer, comprising of both the cortical bone plate and trabecular bone) and layers c and d the trabecular layers. Layers a and b are collectively known as subchondral bone. 3mm, 4mm, 4mm and 4mm sections were sawn respectively at increasing distance from the articular cartilage (refer to Figure 2.3) using a circular saw (Microslice2), to allow independent analysis of the cortical and trabecular layers of the bone.

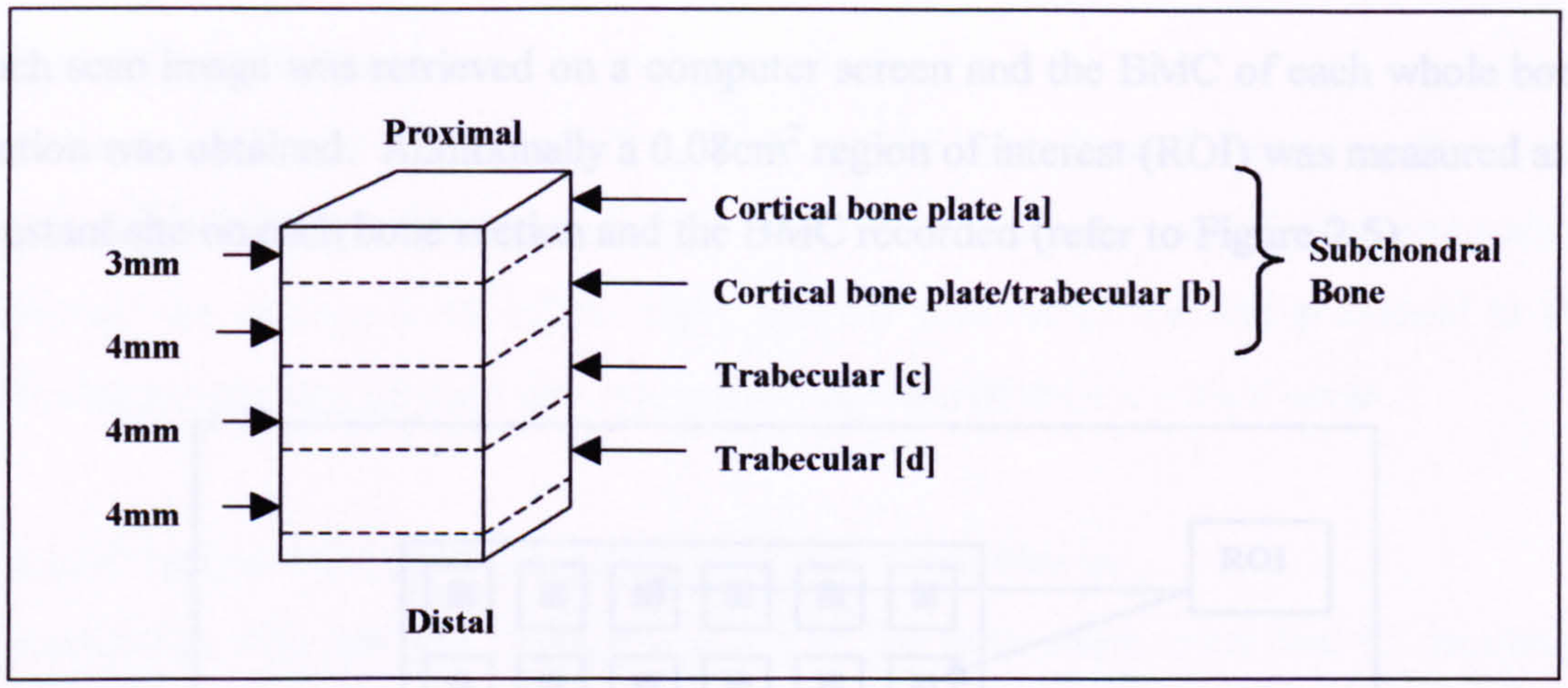


Figure 2.3: Schematic representation of the areas of bone sawn.

2.2.1.4 Dual Energy X-ray Absorptiometry

Quantification of the BMD was attained using Dual Energy X-ray Absorptiometry (DEXA) (Lunar PIXImus densitometer, Lunar Corp.). Each section of bone was thawed to room temperature (approx 25°C) prior to testing. 18 bone sections were placed onto a non-slip dish and scanned in the densitometer with the proximal surface parallel to the horizontal axis of the scan image (refer to Figure 2.4).

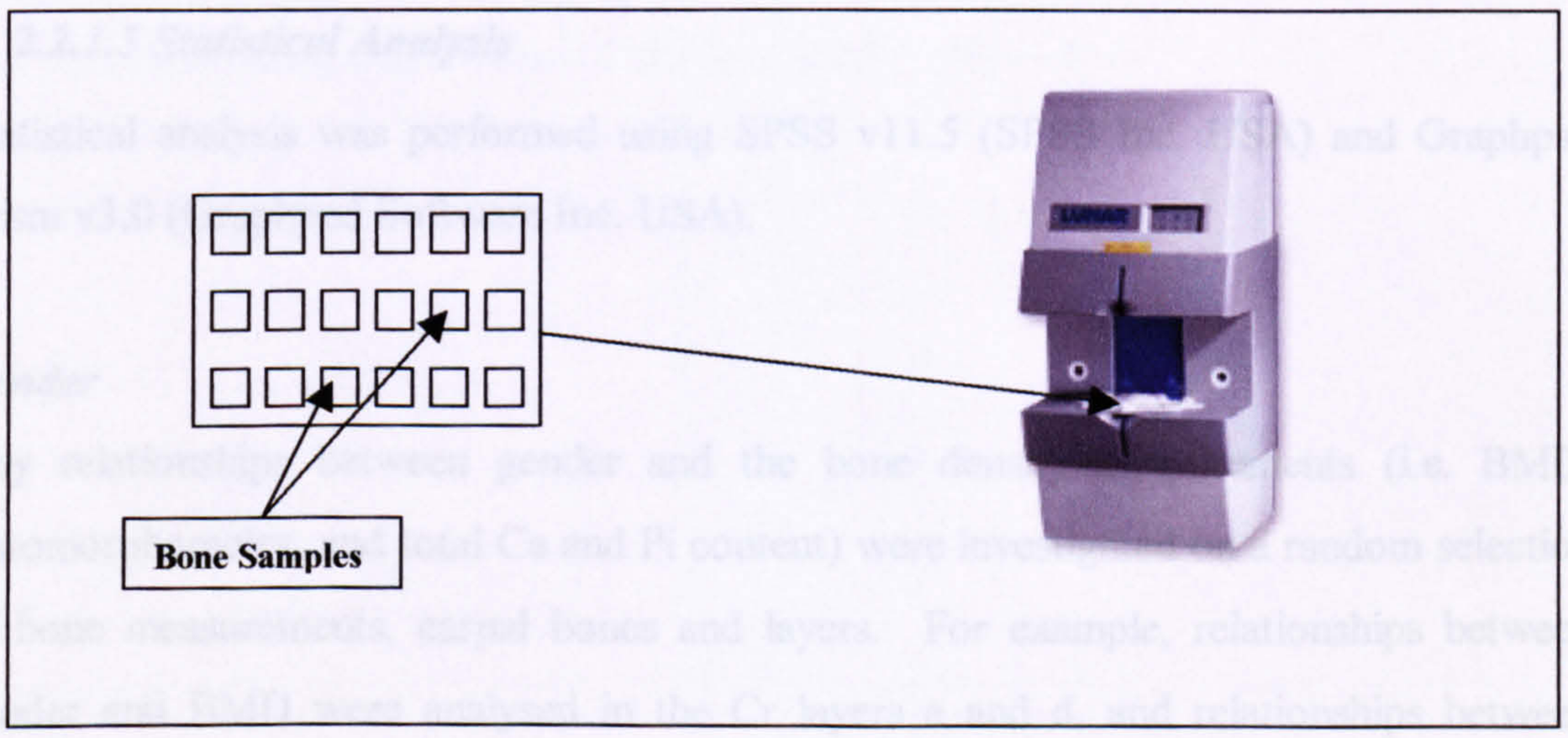


Figure 2.4: Photograph of the Lunar PIXImus (right) and a schematic representation of bone sample in a non-slip dish (left).

Each scan image was retrieved on a computer screen and the BMC of each whole bone section was obtained. Additionally a 0.08cm^2 region of interest (ROI) was measured at a constant site on each bone section and the BMC recorded (refer to Figure 2.5).

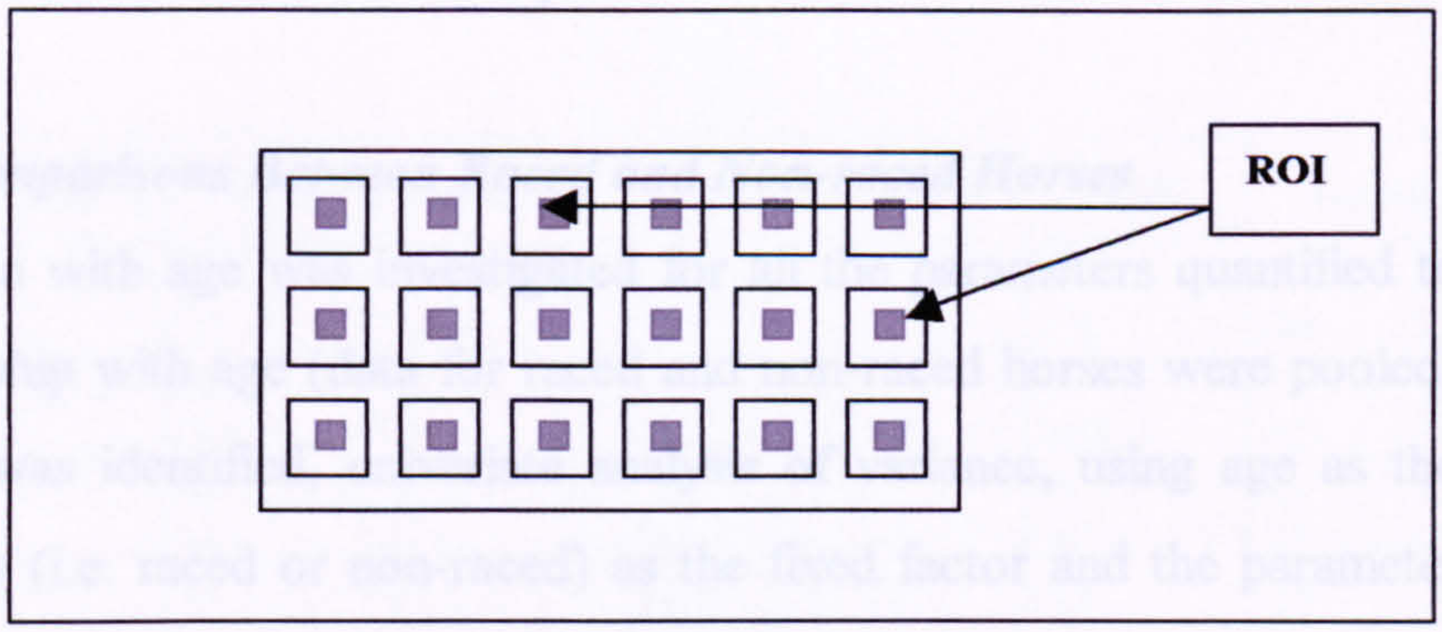


Figure 2.5: Schematic representation of the location of the ROI.

Due to the undulations in the articular surface of bone, the proximodistal thickness of the layer a bone sections were measured with a sliding calliper at 6 sites, to produce an average thickness of the bone. The volumetric BMD was calculated from the surface BMD (calculated from the BMC) divided by the thickness.

2.2.1.5 Statistical Analysis

Statistical analysis was performed using SPSS v11.5 (SPSS Inc. USA) and Graphpad Prism v3.0 (Graphpad Software Inc. USA).

Gender

Any relationships between gender and the bone density measurements (i.e. BMD, histomorphometry, and total Ca and Pi content) were investigated on a random selection of bone measurements, carpal bones and layers. For example, relationships between gender and BMD were analysed in the Cr layers a and d, and relationships between gender and the total Ca and Pi were analysed in the Cr layer c and the C3 layer a. Depending upon the distribution of the data, a parametric test (unpaired t-test) or non-parametric test (Mann-Whitney test) was used to determine any differences. Statistical significance was set at the level of $p<0.05$.

Right and Left Differences

Data for the right and left Cr and C3 of each cadaver was obtained, hence a paired t-test was performed between the left and right values to ascertain any carpal joint asymmetry. However, the average value of the right and left was calculated and presented in the following results section since this represents the value of the individual horse.

Age and Comparisons Between Raced and Non-raced Horses

A correlation with age was investigated for all the parameters quantified to determine any relationship with age (data for raced and non-raced horses were pooled). Where a correlation was identified, univariate analysis of variance, using age as the covariate, racing status (i.e. raced or non-raced) as the fixed factor and the parameter quantified (i.e. BMD) as the dependent variable, was used to determine if age was significantly influencing the parameter quantified regardless of racing status. Statistical significance was set at the level of $p<0.05$. If there was no significant covariation with age, and dependent upon the distribution of the data, a parametric test (unpaired t-test) or non-parametric test (Mann-Whitney test) was subsequently used to determine differences between the racehorses and non-racehorses.

Differences between the various layers of bone (i.e. cortical and trabecular) were additionally analysed. For the parametric analyses, one-way ANOVA was used and for non-parametric analyses, Kruskal-Wallis was used.

2.2.2 Histomorphometry

2.2.2.1.Equine Samples

Table 2.3 shows the horses used for histomorphometrical analysis. Analysis was performed on the left Cr and C3 only of each horse (raced n=11, non-raced n=11).

Raced	Age	Gender	Non-raced	Age	Gender
R1	7	G	**7**	10	M
R2	7	M	**8**	11	M
R3	8	G	**9**	8	G
R4	4	C	**10**	NA	G
R5	6	C	**11**	3	G
R6	7	G	**12**	6	G
R7	7	C	**13**	11	M
R8	7	C	**14**	17	G
R9	6	C	**15**	7	G
R10	6	C	**16**	17	G
R11	7	G	**17**	14	G

Table 2.3: The age (years) and gender of the horses. (C= Colt, F= Filly G= Gelding (neutered male), M=mare, NA= Not Known).

2.2.2.2 Tissue Sample Site

Bone samples were sectioned from adjacent sites to those used for BMD quantification. For the Cr a 0.5mm section was sawn on the lateral adjacent side and for the C3 a 0.5mm section was sawn on the medial adjacent side. The difference in sample site between the Cr and C3 was utilised to avoid obtaining the cortex of the bone. These bone sections were fixed in 70% ethanol (aq) solution and were provided to BIOResults Ltd. (Bristol, UK) for analysis.

2.2.2.3 Tissue Preparation

Bones were dehydrated in a series of ethanol solutions, ascending in concentration from 70-100%. Lipids were removed using chloroform, and the bones infiltrated and embedded in LR White hard acrylic resin (London White Resin Company, UK). The blocks were sanded using a belt sander to remove the potentially damaged bone adjacent to the saw cut, and then mounted in the chuck of a Jung Autocut microtome. Sections

were cut at an indicated thickness of 5μm from the front of the block using a tungsten-carbide tipped D-profile steel knife. The initial sections obtained were discarded to allow trimming into the block, and then a pair of sections were taken and mounted on glass slides. The slides were stained using Goldner's modified trichrome stain to render the mineralised bone green and the unmineralised osteoid red.

2.2.2.4 Histomorphometrical Analysis

Slides were viewed using a Nikon Eclipse E400 microscope, equipped with a Nikon Plan 4x objective lens, a Nikon Plan 0.5x relay and polarising filters. A three-chip colour digital video camera (Sony, Power HAD) mounted on the microscope relayed an image of the specimen to a colour video monitor (Sony, Trinitron Multiscan 400PS). Image analysis was performed using a semi-automated system comprising Osteomeasure bone-image analysis software (Osteometrics Inc., USA), and a digitising bit pad (CalComp drawing board III) employing a CalComp cursor-mouse. Movement of the cursor-mouse on the bit pad superimposed a line tracing on the image of the specimen on the video monitor. In this manner, the number, line lengths and areas of features in the specimen were determined.

2.2.2.5 Sampling Procedure

Due to the large volume of tissue to be analysed, it was necessary to utilise a suitable sampling procedure. The sections consisted of a slice of bone 1cm wide by approximately 3 to 4cms in length. The mid line of each section was determined under the microscope and a series of fields measured adjacent to and either side of the midline. To allow comparison of the BMD and histomorphometry results, the four sampling layers (a-d) established during BMD tissue preparation were identified and measured. Each layer consisted of 6 measuring fields (refer to Figure 2.6)

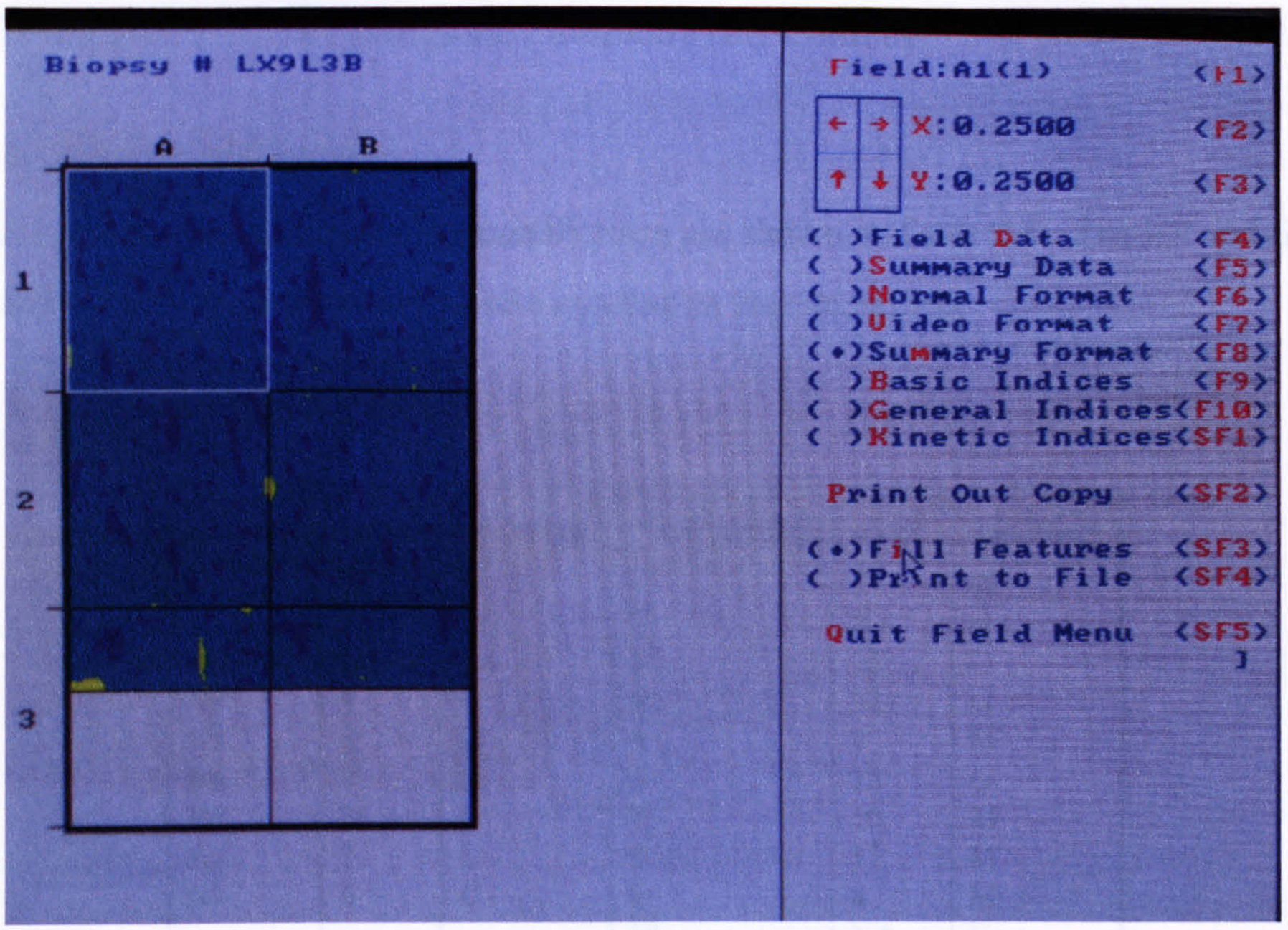


Figure 2.6: To illustrate video monitor screen showing digitised map of one zone, (in this example layer a). Note the six fields analysed extending 3mm from the bottom of the articular cartilage (top of screen). Different features are assigned different colours during measurement to facilitate quality control.

2.2.2.6 Statistical Analysis

Statistical analysis was performed using SPSS v11.5 (SPSS Inc. USA) and Graphpad Prism v3.0 (Graphpad Software Inc. USA), as detailed in section 2.2.1.5.

2.2.3 Calcium (Ca) and Inorganic Phosphate (Pi) Content

2.2.3.1 Equine Samples

The samples used for Ca and Pi quantification are shown in Table 2.4. The left and right Cr and C3 of 9 racehorses and 11 non-racehorses was quantified.

Raced	Age	Gender	Non-raced	Age	Gender
R1	7	G	**1**	17	M
R2	7	M	**2**	NA	G
R3	8	G	**3**	15	G
R4	4	C	**4**	22	G
R5	6	C	**5**	9	M
R6	7	G	**6**	12	G
R7	7	C	**7**	10	M
R8	7	C	**8**	11	M
R9	6	C	**9**	8	M
			10	NA	G
			11	3	G

Table 2.4: The age (years) and gender of the horses. (C= Colt, G= Gelding (neutered male), M=mare, NA= Not Known).

2.2.3.2 Tissue Preparation

The bone sections were snap frozen in liquid nitrogen and reduced to a fine powder using a freezer mill (Spex Mill, UK). A proportion of the pulverised bone used for Ca and Pi quantification was freeze-dried, weighed and hydrolysed in 5mg/ml 6N HCL at 110°C overnight. The bone hydrolysate was then freeze-dried in an acid freeze drier for 24-72hrs until all the acid was removed. The hydrolysate was then resuspended in 1ml dH_2O and vortexed and 100µl aliquots were taken for Ca and Pi quantification.

2.2.3.3 Assay

For total Ca determination, Arsenazo III reagent (Raichem Reagents Applications, Inc. USA) was added to the 100µl aliquot and the colour reaction, which is proportional to the concentration of Ca in the sample, was quantified on an automated analyser (Konelab 30i, Thermo Clinical Labsystems, Finland). Determination of Pi was attained via the

same method except ammonium molybdate reagent (Kone Diagnostics, USA) was used to form the colour reaction. The total amount of Ca and Pi was expressed as a percentage of dry bone weight.

2.2.3.4 Statistical Analysis

Statistical analysis was performed using SPSS v11.5 (SPSS Inc. USA) and Graphpad Prism v3.0 (Graphpad Software Inc. USA), as detailed in section 2.2.1.5.

2.3 RESULTS

2.3.1 Age and Gender

2.3.1.1 Age

a) BMD:

The ages of the horses used to quantify the BMD varied from 3 to 17 years, with the widest age range being evident in the non-racehorses. The mean ages of the horses are as follows:

- Raced: Mean age= 6; SEM= 0.3.
- Non-raced: Mean age= 10; SEM= 1.4.

b) Histomorphometry:

The ages of the horses used for histomorphometry analysis varied from 3 to 17 years. The mean ages of the horses are as follows:

- Raced: Mean age= 6; SEM= 0.3.
- Non-raced: Mean age= 10; SEM= 1.4.

c) Ca and Pi:

The ages of the horses used to quantify the Ca and Pi content varied from 3 to 17 years. The mean ages of the horses are as follows:

- Raced: Mean age= 6; SEM= 0.3.
- Non-raced: Mean age= 11; SEM= 1.8.

2.3.1.2 Gender

To avoid confusion the number of colts and geldings has been combined and will be referred to as males. The mares will be referred to as females. Additionally, the number of males and females in the raced and non-raced horses has been combined to represent the total number of males and the total number of females in the study.

a) BMD – Region of interest (Cr layers a and d):

There was no significant difference in the BMD in layer a (p=0.70) of the Cr between males (1.16+/-0.02) and females (1.18+/-0.03) and layer d (p=0.53) between males (0.709 +/-0.02) and females (0.747 +/-0.05).

b) Histomorphometry – Bone area (%) (C3 layers b and c):

There was no significant difference in the percentage bone area in layer b (p=0.65) of the Cr between males (79.2+/-2.5) and females (76.8+/-4.8) and layer c (p=0.36) between males (66.8 +/-3.09) and females (61.1 +/-4.6).

c) Ca and Pi (Cr layer c and C3 layer a):

There was no significant difference in the Ca and Pi content in layer c of the Cr (p=0.68) between males (51.7+/-1.8) and females (50.3+/-2.9) and layer a of the C3 (p=0.58) between males (54.9+/-1) and females (56.01+/-1.7).

2.3.2 Bone Mineral Density (BMD)

2.3.2.1 BMD of the Whole Bone Section

1) Cr:

a) Right and left differences

The right and left values for the all the results in this thesis are given in Appendix Three.

There was a significant difference between the right (0.68+/-0.03) and left (0.72+/-0.04) BMD values in layer c ($p=0.04$) and between the right (0.6+/-0.03) and left (0.63+/-0.03) BMD values in layer d ($p=0.04$) in the non-raced Cr. There were no significant differences in the values of the raced right and left Cr (see Appendix Three).

b) Correlation with age

The BMD of layers a to d of the Cr did not significantly correlate with age; layer a ($r^2= -0.06$, $p=0.23$), b ($r^2= -0.0007$, $p=0.91$), c ($r^2= -0.02$, $p=0.48$) and d ($r^2= -0.00006$, $p=0.97$).

c) Comparisons between raced and non-raced horses

The volumetric BMD was not significantly different in layers a to d of the Cr between the raced and non-raced horses (refer to Figure 2.7).

d) Pattern of the BMD throughout the depth of the bone

The pattern of the BMD throughout layers a to d in both the raced and non-raced horses is shown in Figure 2.7. The pattern is similar in both groups of horses, decreasing from layers a to d. The variation amongst layers a to d is significant ($p<0.0001$).

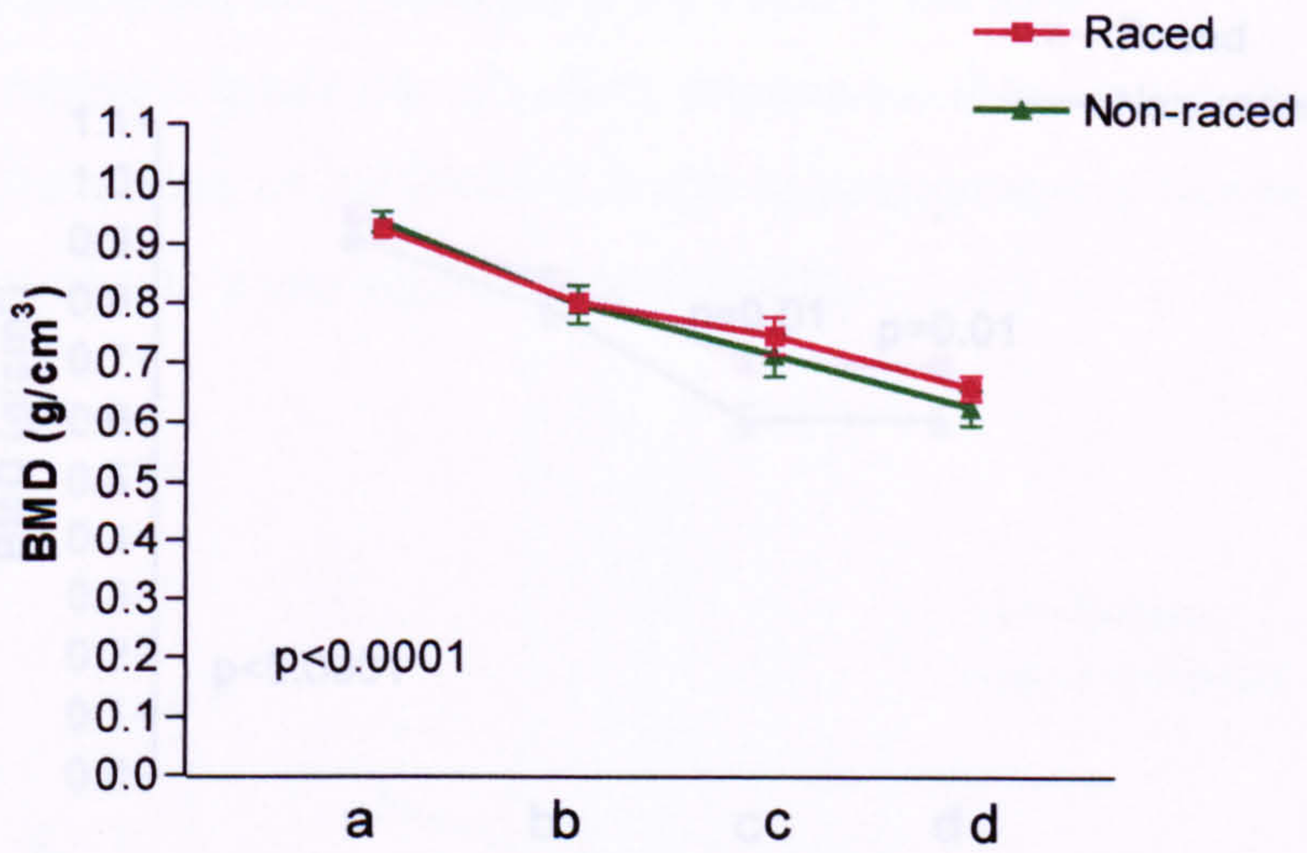


Figure 2.7: The BMD of the whole bone section in the various layers of the Cr. The variation amongst layers a to d was significant ($p<0.0001$).

2) C3:

a) Right and left differences

There was a significant difference between the right (0.93+/-0.01) and left (0.94+/-0.008) BMD values in layer a ($p=0.04$) of the raced C3. However, there were no differences in the non-raced C3 (see Appendix Three).

b) Correlation with age

The BMD of layers a to d of the C3 did not significantly correlate with age; layer a ($r^2= -0.00007$, $p=0.96$), b ($r^2= -0.03$, $p=0.37$), c ($r^2= -0.15$, $p=0.06$) and d ($r^2= -0.15$, $p=0.06$).

c) Comparisons between raced and non-raced horses

The volumetric BMD was greater in all layers of the C3 of the racehorses compared to the non-racehorses, reaching significance in layers c and d (refer to Figure 2.8).

d) Pattern of the BMD throughout the depth of the bone

The pattern of the BMD throughout the layers a to d is similar in both the raced and non-raced horses (refer to Figure 2.8). The variation amongst the layers was significant ($p<0.0001$), decreasing from layers a to c, from which point the BMD reaches a plateau.

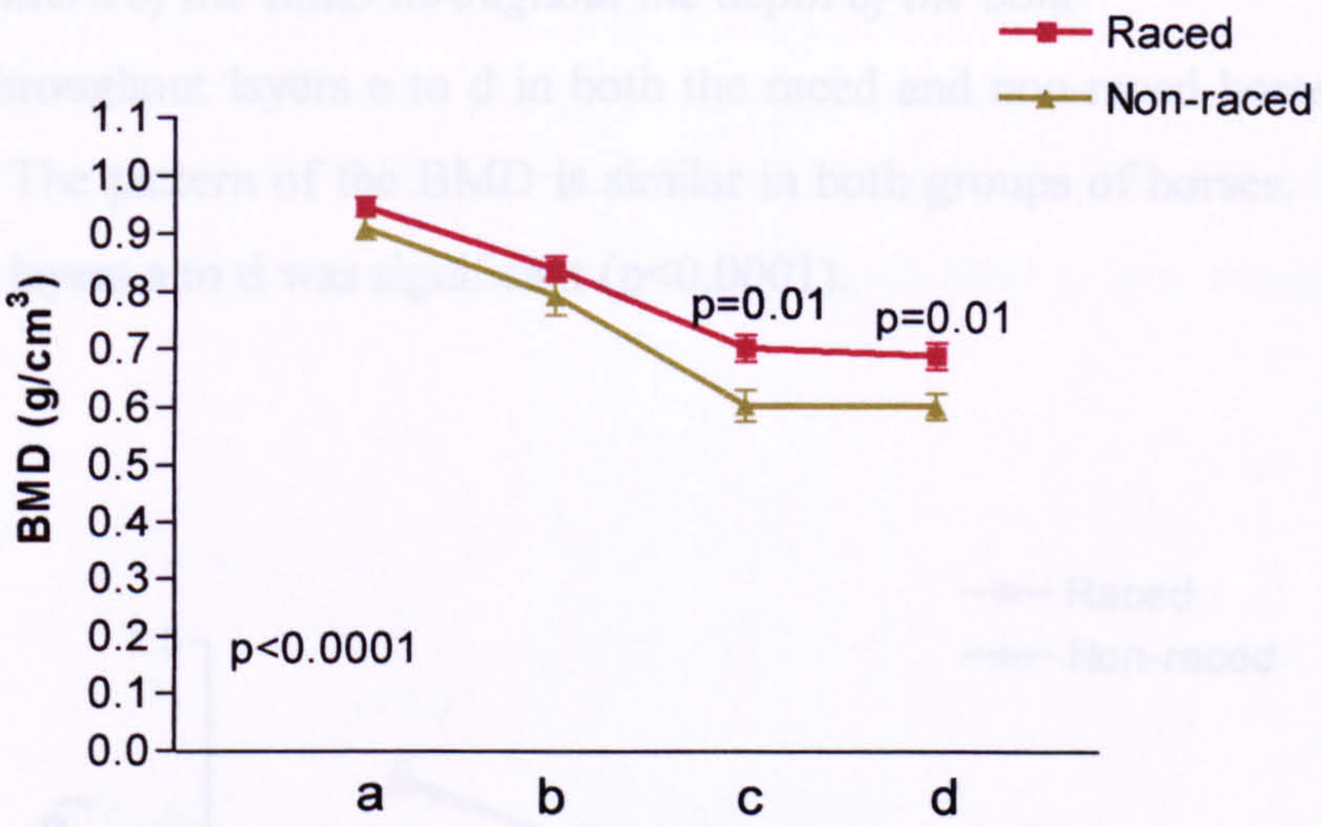


Figure 2.8: The BMD of the whole bone section in the various layers of the C3. There was a significant difference between the raced and non-raced horses in layers c ($p=0.01$) and d ($p=0.01$). The variation amongst the layers a to d was significant ($p<0.0001$).

2.3.2.2 BMD of the Region of Interest (ROI)

1) Cr:

a) Right and left differences

There was no significant difference between the right and left BMD ROI values in either the raced or non-raced Cr (see Appendix Three).

b) Correlation with age

The BMD (ROI) of layers a to d of the Cr did not significantly correlate with age; layer a ($r^2= 0.11$, $p=0.12$), b ($r^2= -0.007$, $p=0.68$), c ($r^2= -0.01$, $p=0.56$) and d ($r^2= -0.002$, $p=0.81$).

c) Comparisons between raced and non-raced horses

The BMD was not significantly different in layers a to d of the raced compared to non-raced horses (refer to Figure 2.9).

d) Pattern of the BMD throughout the depth of the bone

The BMD throughout layers a to d in both the raced and non-raced horses is shown in Figure 2.9. The pattern of the BMD is similar in both groups of horses. The variation amongst the layers a to d was significant ($p<0.0001$).

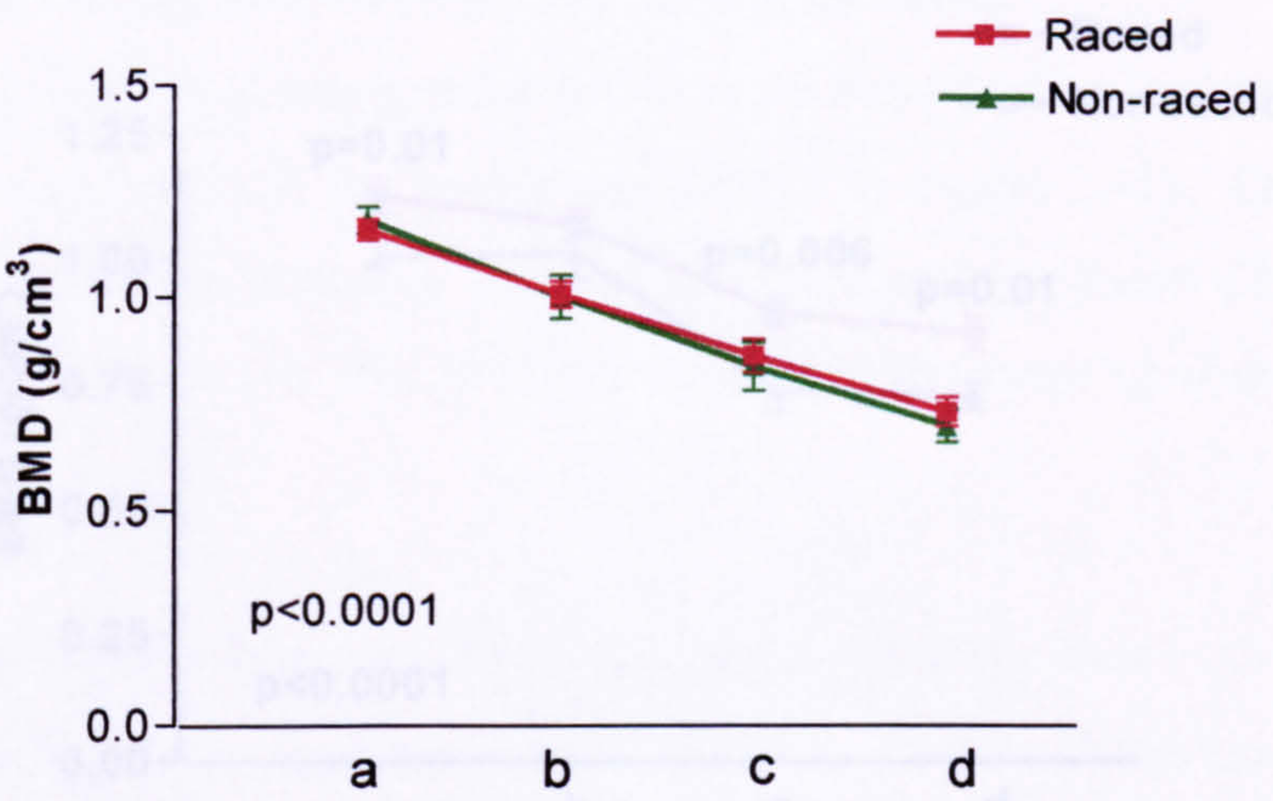


Figure 2.9: The BMD of the ROI in the various layers of the Cr. The variation amongst the layers a to d was significant ($p<0.0001$).

2) C3:

a) Right and left differences

There was no significant difference between the right and left BMD ROI values in either the raced or non-raced C3 (see Appendix Three).

b) Correlation with age

The BMD (ROI) of layers a to d of the C3 did not significantly correlate with age; layer a ($r^2= -0.10$, $p=0.12$), b ($r^2= -0.04$, $p=0.31$), c ($r^2= -0.15$, $p=0.06$) and d ($r^2= -0.12$, $p=0.08$).

c) Comparisons between raced and non-raced horses

The BMD was significantly greater in layers a, c and d of the raced compared to the non-raced horses (refer to Figure 2.10).

d) Pattern of the BMD throughout the depth of the bone

Figure 2.10 shows the BMD throughout the layers a to d in both the raced and non-raced horses. The pattern of the BMD is similar in both the groups of horses, additionally, the variation amongst the layers a to d was significant ($p<0.0001$), decreasing from layers a to d.

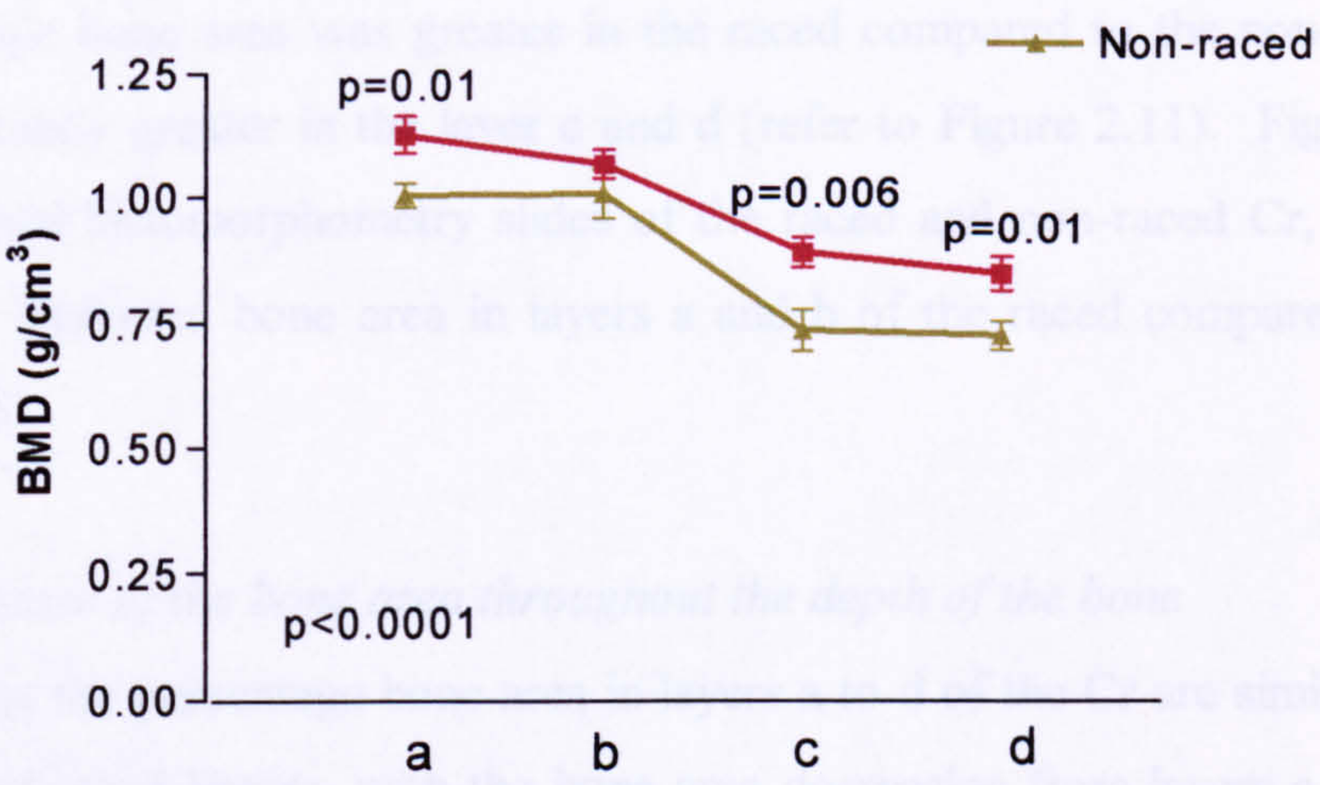


Figure 2.10: The BMD of the ROI in the various layers of the C3. There was a significant difference between the raced and non-raced horses in layers a ($p=0.01$), c ($p=0.006$) and d ($p=0.01$). The variation amongst the layers a to d was significant ($p<0.0001$).

2.3.3 Histomorphometry

Due to freeze thawing, it was not possible to determine any cell based parameters; nor to distinguish osteoid from necrotic and pyknotic cellular materials. Evaluation of trabecular number, width or separation was not possible because the objective criteria for the definition of a trabeculum could not be established in this bone type. Hence it was only possible to evaluate bone area and bone perimeter in the specimens.

2.3.3.1 Bone Area (B.Ar. /T.Ar %)

The bone area (B.Ar.) values were normalised against tissue area (T.Ar.) and expressed as a percentage (%) (B.Ar. /T.Ar %).

1) Cr:

a) Correlation with age

The percentage bone area of layers a to d of the Cr did not significantly correlate with age; layer a (r^2= -0.01, p=0.58), b (r^2= -0.13, p=0.12), c (r^2= -0.12, p=0.14) and d (r^2= -0.13, p=0.11).

b) Comparisons between raced and non-raced horses

The percentage bone area was greater in the raced compared to the non-raced horses, being significantly greater in the layer c and d (refer to Figure 2.11). Figures 2.12 and 2.13 are typical histomorphometry slides of the raced and non-raced Cr, which clearly illustrate the increased bone area in layers a and b of the raced compared to the non-raced horses.

c) Pattern of the bone area throughout the depth of the bone

The pattern of the percentage bone area in layers a to d of the Cr are similar in both the raced and non-raced horses, with the bone area decreasing from layers a to d (refer to Figure 2.11). The variation amongst the layers was significant (p<0.0001).

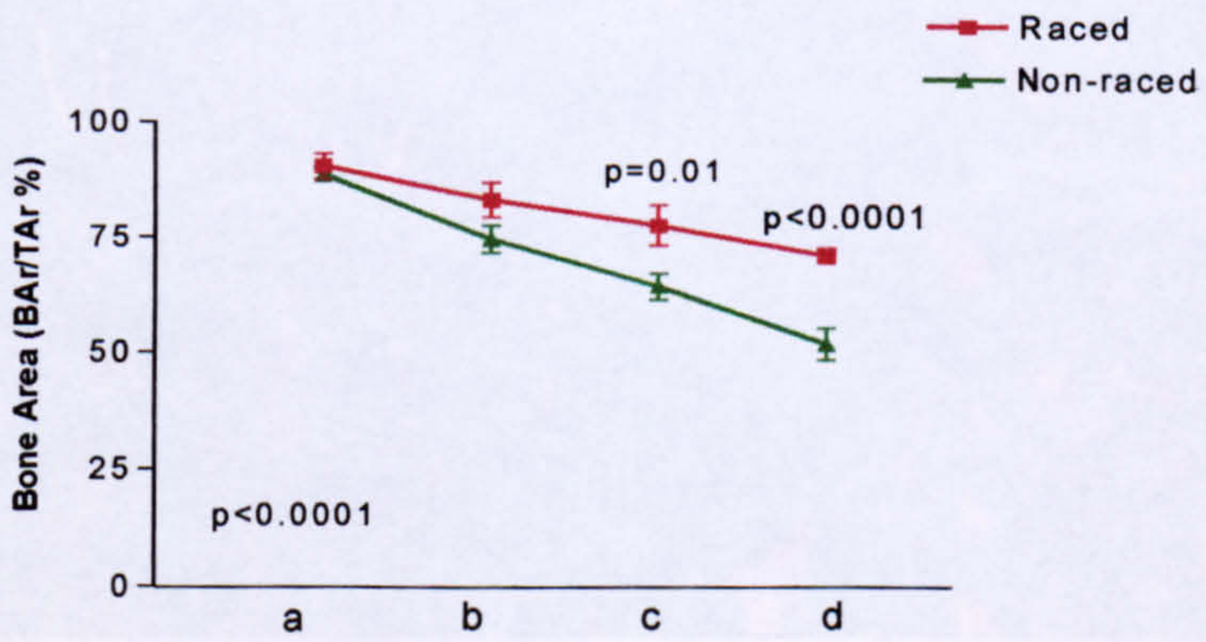


Figure 2.11: The percentage bone area of the various layers of the Cr of the raced and non-raced horses. There was a significant difference between raced and non-raced horses in layer c (p=0.01) and d (p<0.0001). The variation amongst the layers was significant (p<0.0001).

2) C3:

There were no significant correlations with age or significant differences between the raced and non-raced horses in layers a to d of the C3.

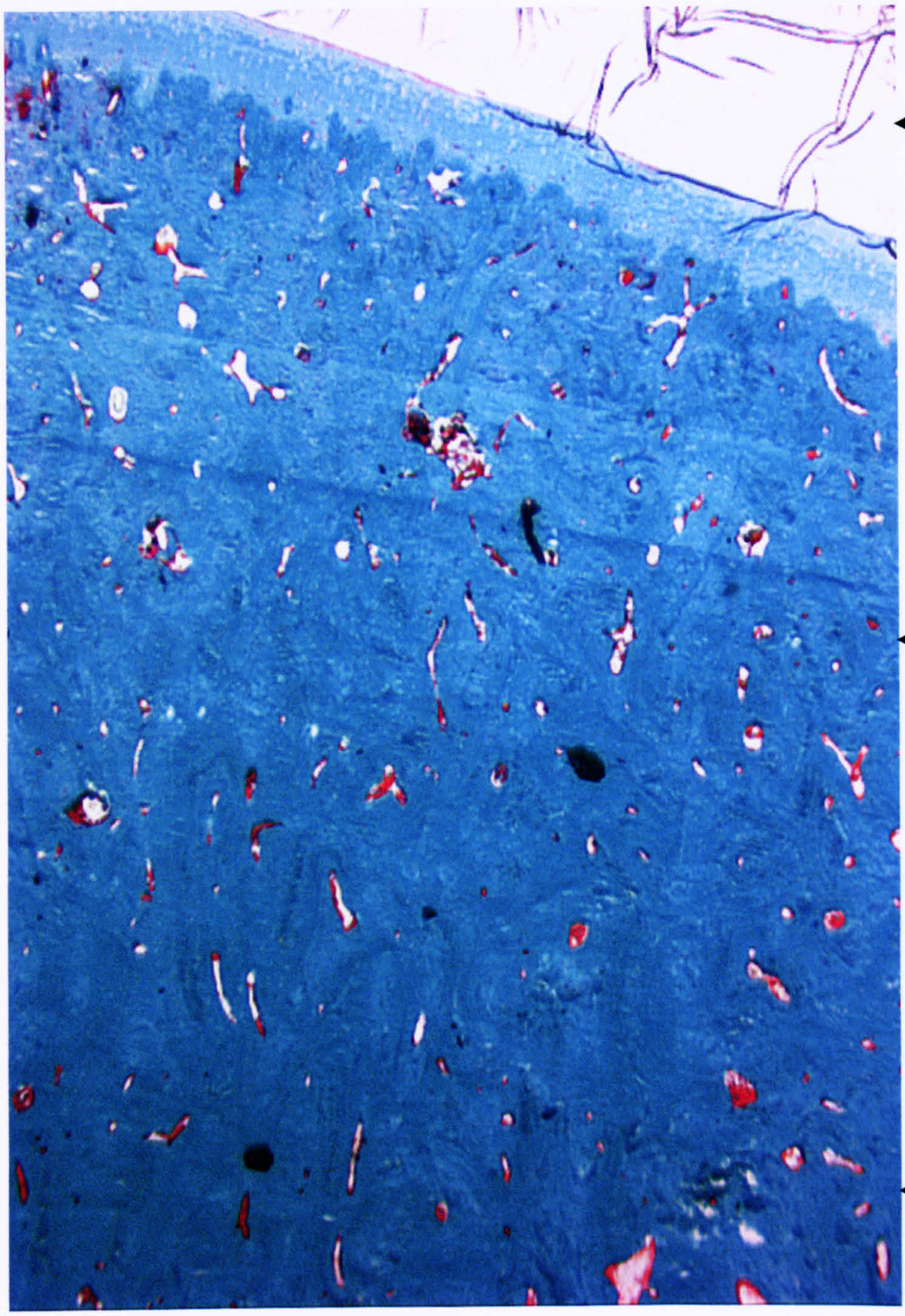


Figure 2.12: Histomorphometry slide of a typical racehorse radial carpal bone specimen. Magnification x2.5. Goldner's modified trichrome stain.

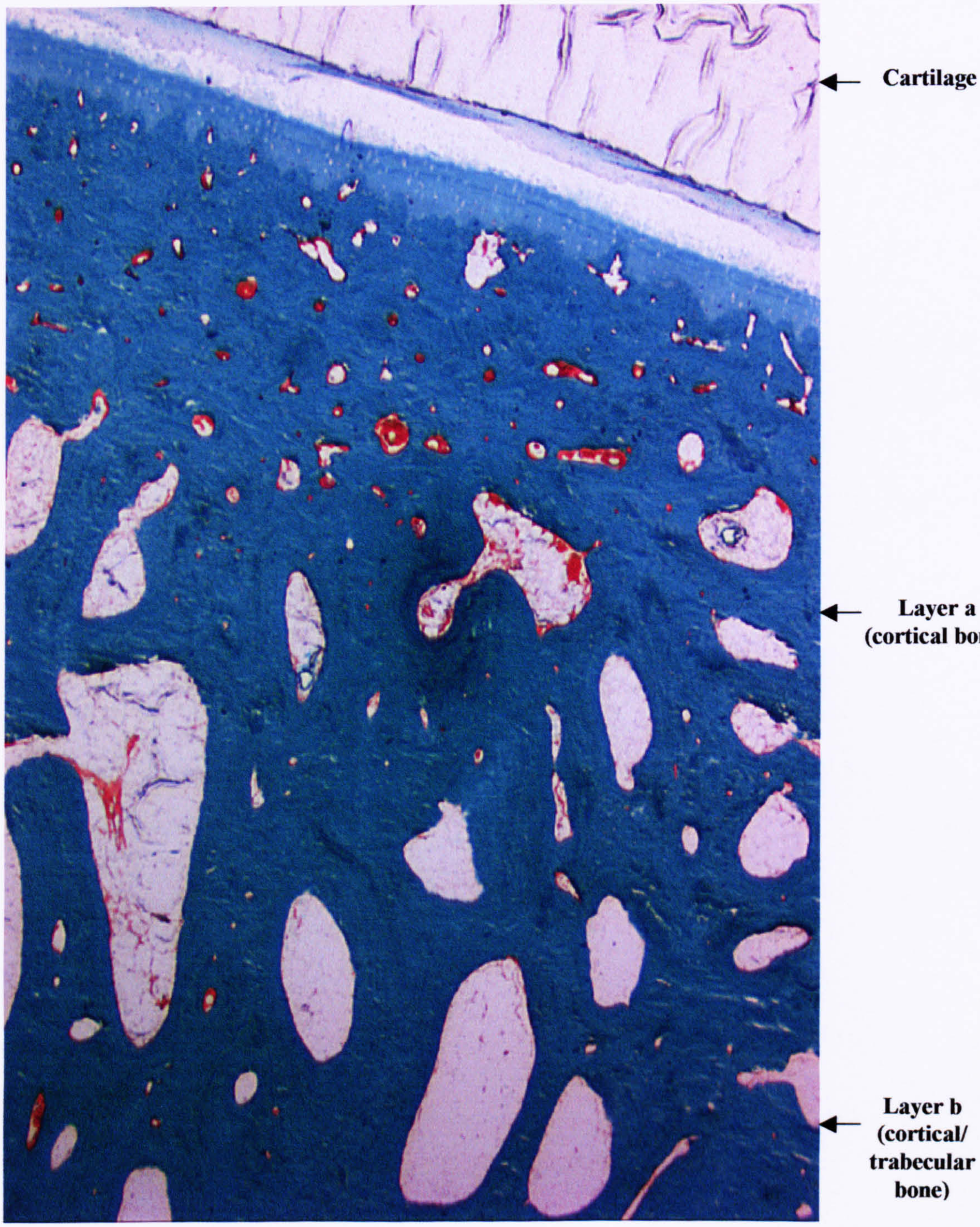


Figure 2.13: Histomorphometry slide of a typical non-racehorse radial carpal bone specimen. Magnification x2.5. Goldner's modified trichrome stain.

2.3.3.2 Bone Perimeter (B.Pm./T.Ar mm^{-1})

The bone perimeter (B.Pm) values were normalised against tissue area (T.Ar) and expressed as mm^{-1} (B.Pm./T.Ar mm^{-1}).

1) Cr:

a) Correlation and covariation with age

The bone perimeter (B.Pm./T.Ar mm^{-1}) values positively correlated with age in layers a, b and c of the Cr (refer to Table 2.5), however, there was only a significant covariation with age in layer b ($p=0.02$).

Layer	Linear (Pearson) Correlation (r^2)	P Value
Bone Perimeter (B.Pm./T.Ar mm^{-1})		
a	+0.33	**0.009**
b	+0.43	**0.002**
c	+0.24	**0.03**
d	+0.11	0.14

Table 2.5: Correlation coefficients of the bone perimeter per tissue area and age in the Cr layers a-d.

b) Comparisons between raced and non-raced horses

Figure 2.14 shows the bone perimeter per tissue area in the Cr layers a to d. There was a significant difference between the raced and non-raced horses in layers a and d, being greatest in the non-raced.

c) Pattern of the bone perimeter (tissue area) throughout the depth of the bone

The pattern of the bone perimeter throughout layers a to d of the Cr are similar in both the raced and non-raced horses, with the bone perimeter decreasing from layers a to c and then increasing from layers c to d (refer to Figure 2.14). The variation amongst the layers was significant ($p<0.0001$).

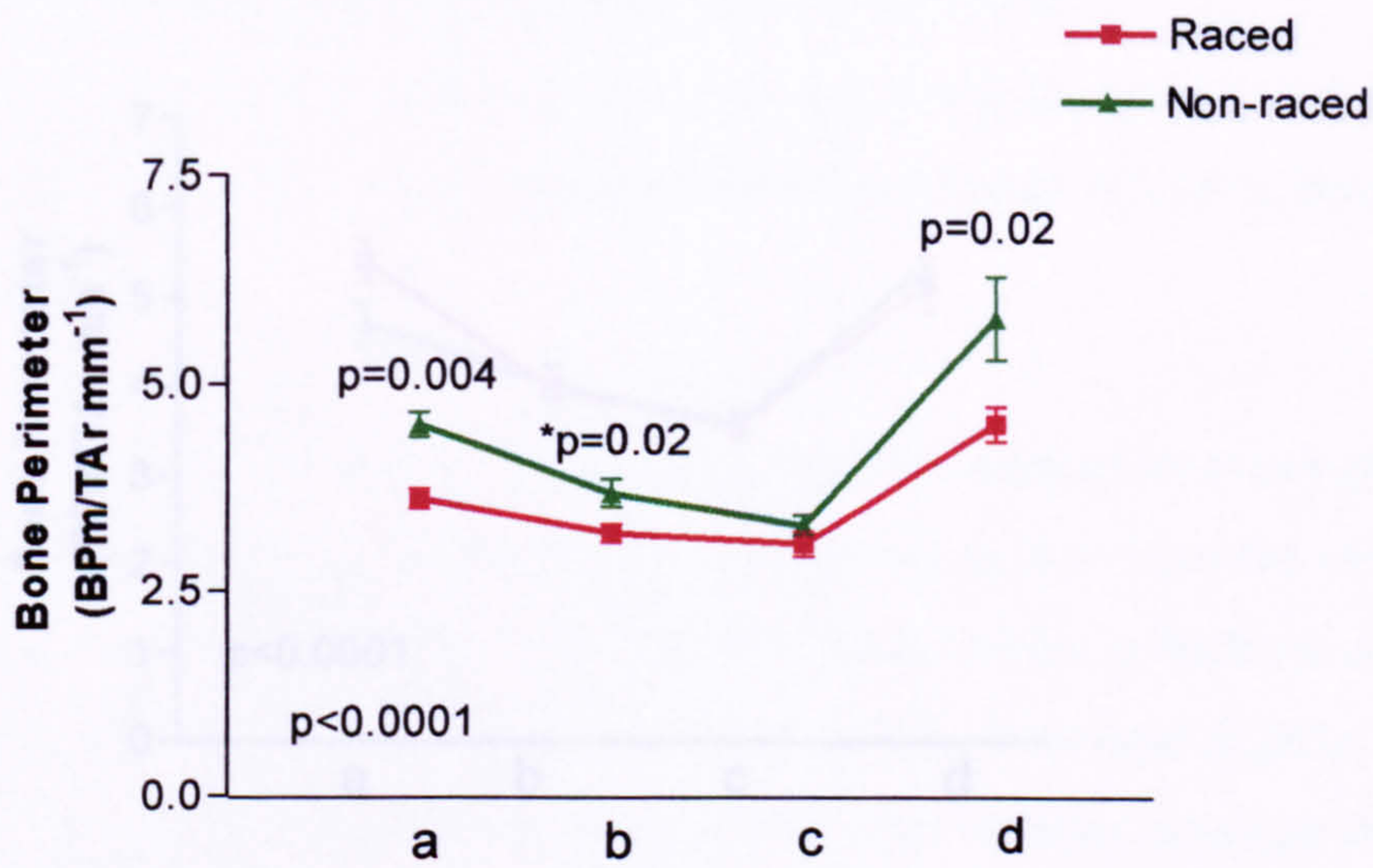


Figure 2.14: The bone perimeter per tissue area of the various layers of the Cr. There was a significant difference between the raced and non-raced horses in layers a (p=0.004) and d (p=0.02). The variation amongst the layers was significant (p<0.0001). Note: *p represents the p value given when there was a significant covariation with age.

2) C3:

a) Correlation with age

The bone perimeter (B.Pm./T.Ar mm^{-1}) of layers a to d of the C3 did not significantly correlate with age; layer a (r^2= -0.04, p=0.39), b (r^2= -0.0002, p=0.94), c (r^2= -0.007, p=0.71) and d (r^2= 0.05, p=0.37).

b) Comparisons between raced and non-raced horses

The bone perimeter was not significantly different in layers a to d of the raced compared to the non-raced C3 (refer to Figure 2.15).

c) Pattern of the bone perimeter (tissue area) throughout the depth of the bone

The pattern of the bone perimeter throughout layers a to d in both the raced and non-raced horses is shown in Figure 2.15. The pattern is similar in both groups of horses, with the bone area decreasing from layers a to c and then increasing from layers c to d. The variation amongst the layers was significant (p<0.0001).

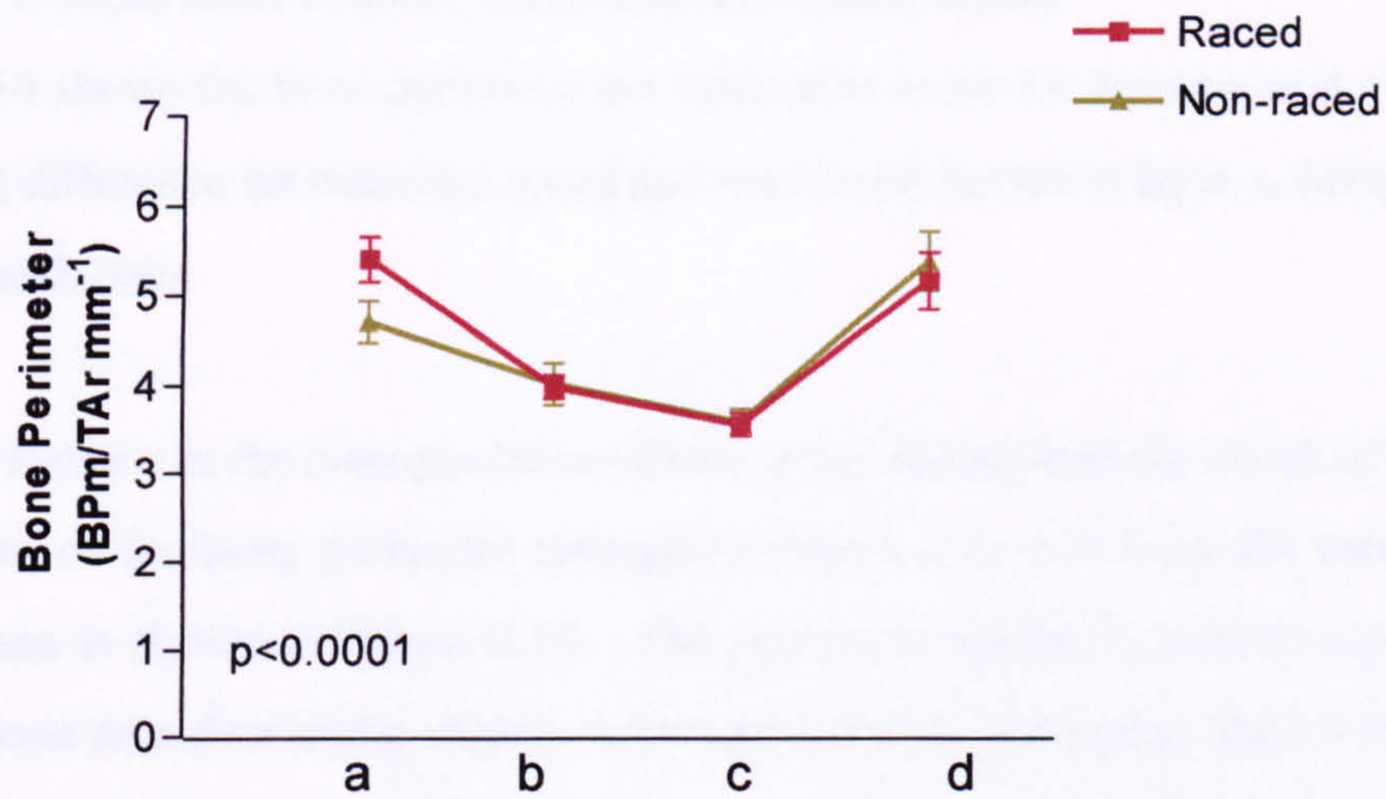


Figure 2.15: The bone perimeter per tissue area of the various layers of the C3. The variation amongst the layers was significant ($p<0.0001$).

2.3.3.3 Bone Perimeter (B.Pm./B.Ar mm^{-1})

The bone perimeter (B.Pm) values were additionally normalised against bone area (B.Ar) and expressed as mm^{-1} (B.Pm./B.Ar mm^{-1}).

1) Cr:

a) Correlation and covariation with age

The bone perimeter (B.Pm./B.Ar mm^{-1}) values positively correlated with age in layers a, b and c of the Cr (refer to Table 2.6). There was a significant covariation with age in layers b ($p=0.01$) and c ($p=0.02$)

Layer	Linear (Pearson) Correlation (r^2)	P Value
Bone Perimeter (B.Pm./B.Ar mm^{-1})		
a	+0.35	**0.007**
b	+0.49	**0.0008**
c	+0.47	**0.001**
d	+0.00001	0.98

Table 2.6: Correlation coefficients of the bone perimeter per bone area and age in the Cr layers a-d.

b) Comparisons between raced and non-raced horses

Figure 2.16 shows the bone perimeter per bone area in the Cr layers a to d. There was a significant difference between the raced and non-raced horses in layer a, being greatest in the non-racehorses.

c) Pattern in the bone perimeter (bone area) throughout the depth of the bone

The pattern of the bone perimeter throughout layers a to d in both the raced and non-raced horses is shown in Figure 2.16. The pattern is similar in both groups of horses, with the bone area decreasing slightly from layers a to b, increasing slightly from layers b to c and then decreasing again from layers c to d. The variation amongst the layers was significant ($p<0.0001$).

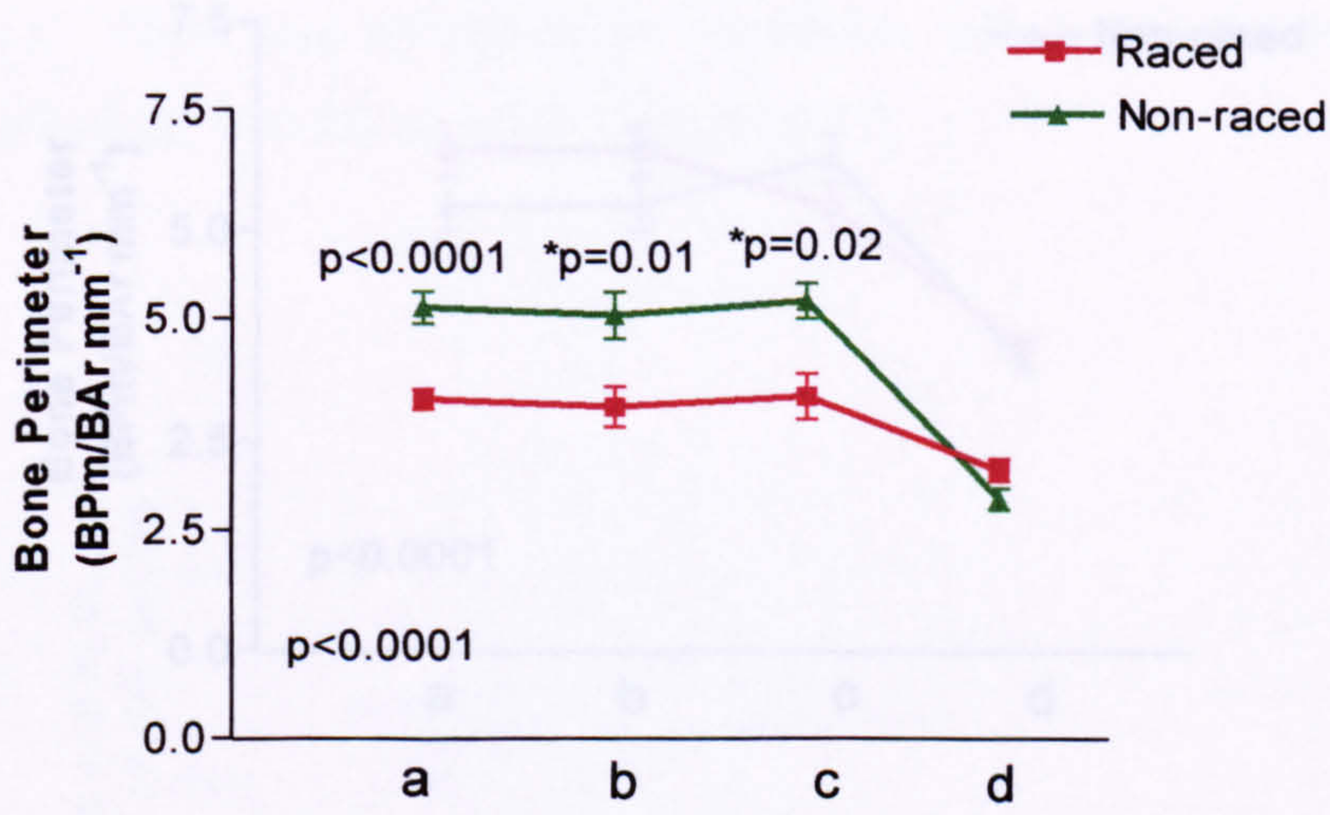


Figure 2.16: The bone perimeter per bone area of the various layers of the Cr. There was a significant difference between the raced and non-raced horses in layer a ($p<0.0001$). The variation amongst the layers was significant ($p<0.0001$). Note: *p represents the p value given where there was a significant covariation with age.

2) C3:

a) Correlation with age

The bone perimeter (B.Pm./B.Ar mm^{-1}) of layers a to d of the C3 did not significantly correlate with age; layer a ($r^2= -0.04$, $p=0.83$), b ($r^2= 0.08$, $p=0.20$), c ($r^2= 0.02$, $p=0.49$) and d ($r^2= -0.01$, $p=0.62$).

b) Comparisons between raced and non-raced horses

The bone perimeter was not significantly different in layers a to d of the raced compared to the non-raced C3 (refer to Figure 2.17).

c) Pattern in the bone perimeter (bone area) throughout the depth of the bone

Figure 2.17 shows the pattern of the bone perimeter in layers a to d of the C3. The pattern varies in the raced and non-raced horses, with the bone perimeter in the raced declining from layers a to d and the bone perimeter in the non-raced horses declining from layers a to b and subsequently increasing form layers b to c. The variation amongst the layers was significant ($p<0.0001$).

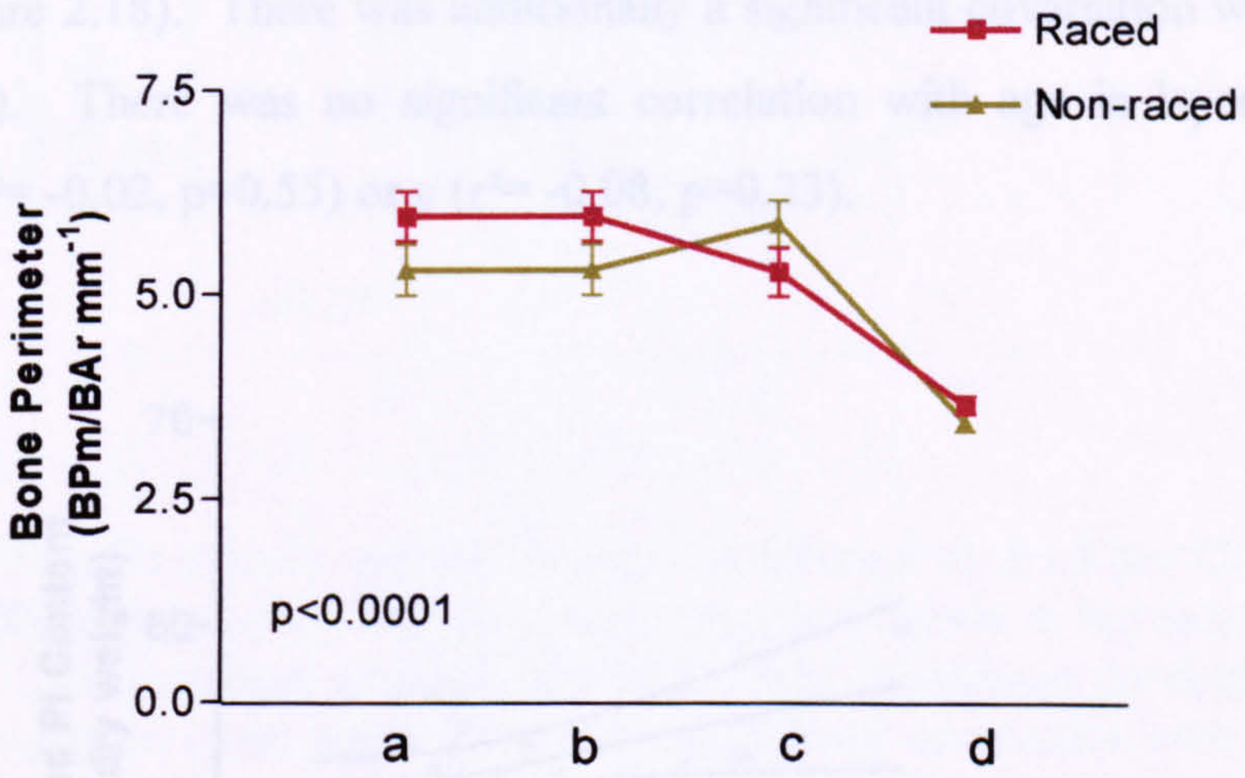


Figure 2.17: The bone perimeter per bone area of the various layers of the C3. The variation amongst the layers was significant ($p<0.0001$).

2.3.4 Calcium and Inorganic Phosphate Content

Within the following results section the combined Ca and Pi content will be expressed as a percentage of tissue dry weight.

1) Cr:

a) Right and left differences

There was a significant difference between the right (55.7+/-2.1) and left (48.86+/-3.79) Ca and Pi content in layer a ($p=0.03$) of the non-raced Cr but there were no differences in the raced Cr (see Appendix Three).

b) Correlation and covariation with age

The Ca and Pi content was found to correlate positively with age in layer d of the Cr (refer to Figure 2.18). There was additionally a significant covariation with age in layer d ($p=0.0001$). There was no significant correlation with age in layers a ($r^2= 0.07$, $p=0.26$), b ($r^2= -0.02$, $p=0.55$) or c ($r^2= -0.08$, $p=0.23$).

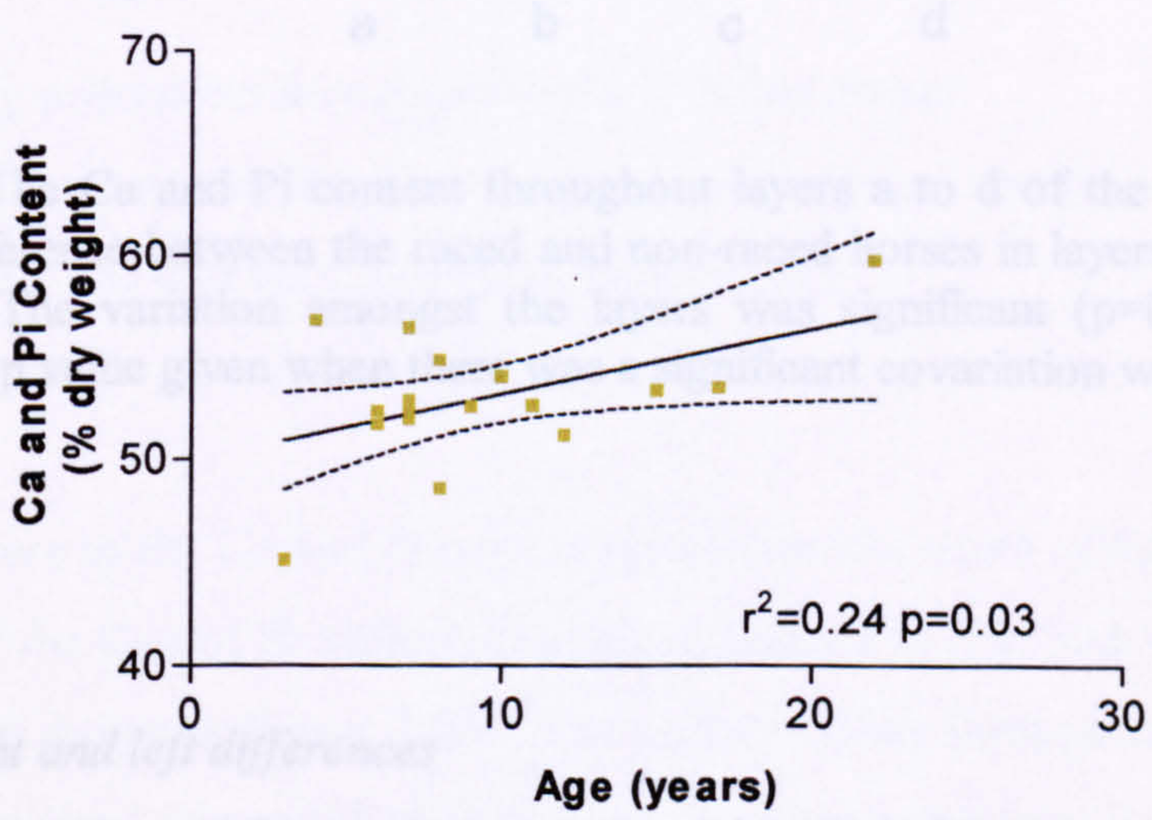


Figure 2.18: The correlation between age and the Ca and Pi content in layer d of the Cr.

c) Comparisons between raced and non-raced horses

The Ca and Pi content was greater in the Cr layers a to d of the raced compared to the non-raced horses, being significantly greater in layers b and c (refer to Figure 2.19).

d) Pattern of the Ca and Pi content throughout the depth of the bone

Figure 2.19 shows the pattern of the Ca and Pi content throughout the layers a to d in both the raced and non-raced horses. The pattern differs throughout the depth of the bone in both groups of horses; in the racehorses the content increases slightly from layer a to b, and subsequently decreases from layers c to d, whereas in the non-racehorses, the content declines from layers a to c and then increases from layers c to d. The variation amongst the layers is significant (p=0.001).

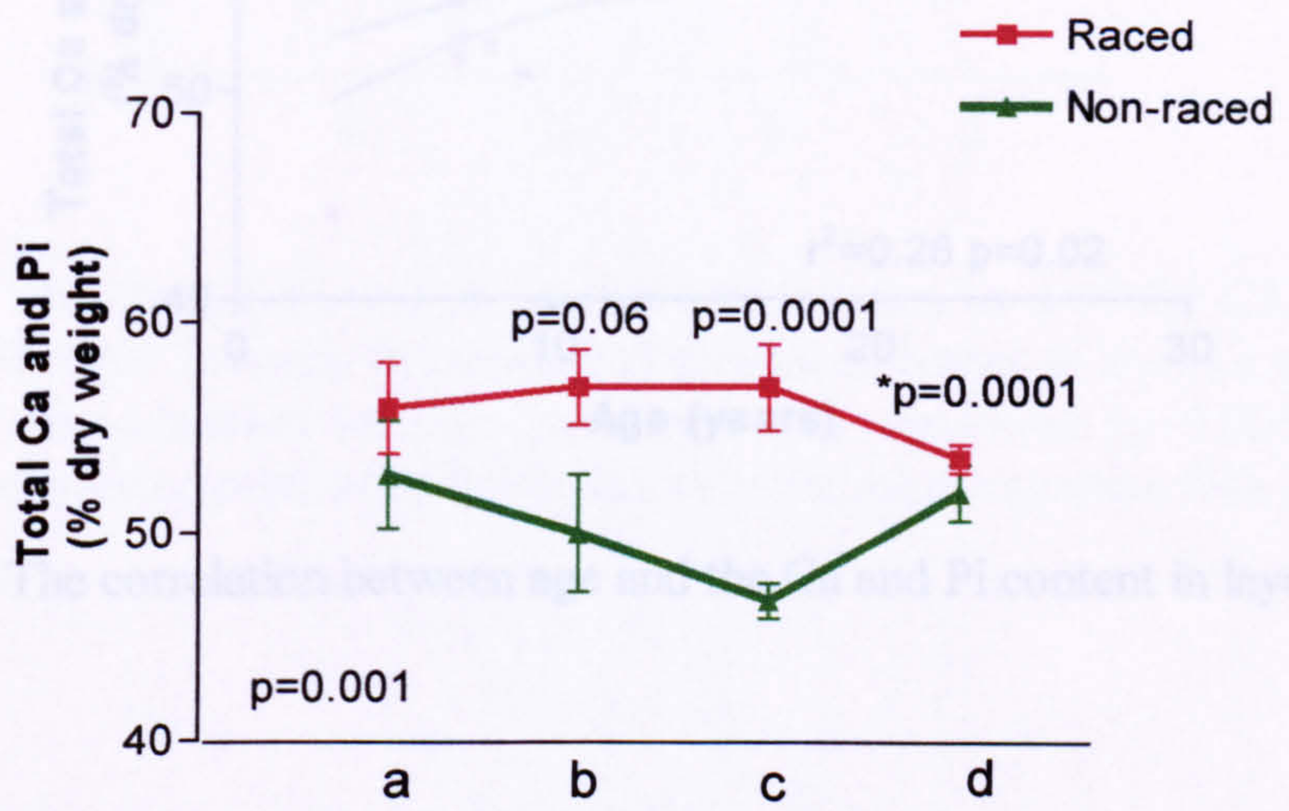


Figure 2.19: The Ca and Pi content throughout layers a to d of the Cr. There was a significant difference between the raced and non-raced horses in layers b (p=0.06) and c (p=0.0001). The variation amongst the layers was significant (p=0.001). Note: *p represents the p value given when there was a significant covariation with age.

2) C3:

a) Right and left differences

There was a significant difference between the right (45.5+/-0.83) and left (45.2+/-0.86) Ca and Pi content in layer c (p=0.005) of the non-raced C3, however, there were no differences in the raced C3 (see Appendix Three).

b) Correlation with age

The Ca and Pi content was found to positively correlate with age in layer a of the C3 (refer to Figure 2.20). There was additionally a significant covariation with age in layer a

(p=0.01). There was no significant correlation with age in layers b (r^2= -0.001, p=0.88), c (r^2= -0.14, p=0.12) or d (r^2= -0.0001, p=0.95).

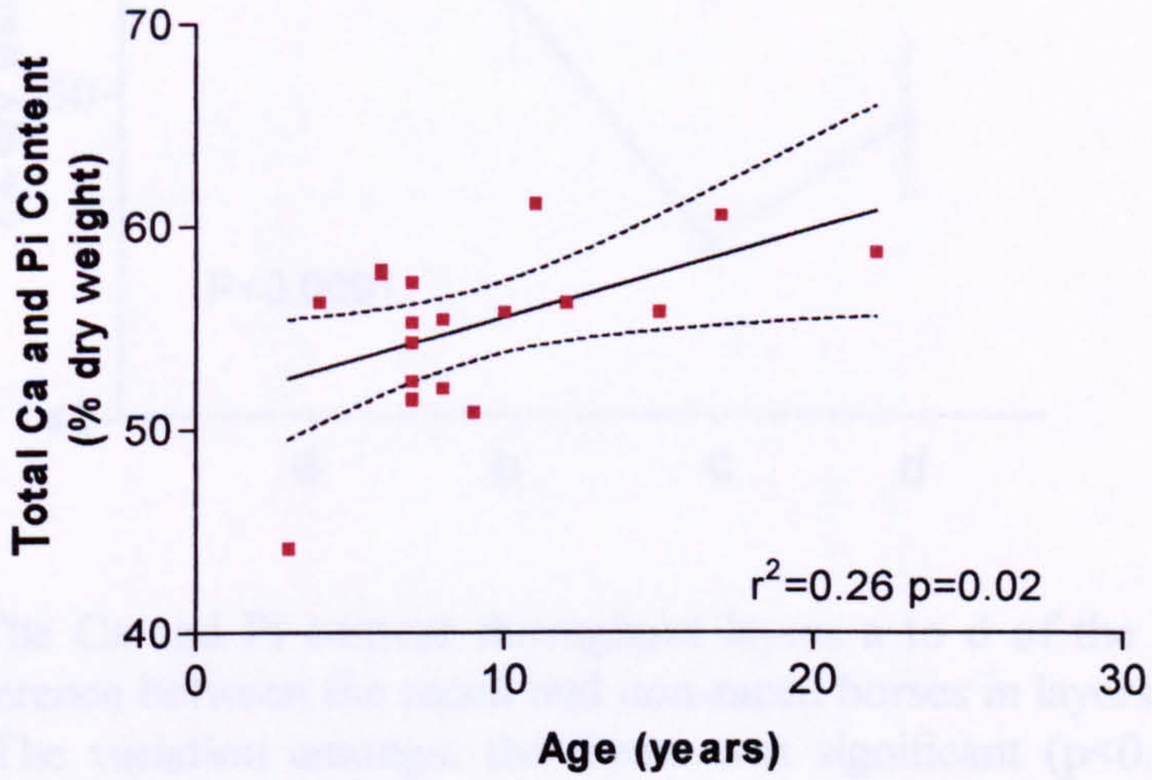


Figure 2.20: The correlation between age and the Ca and Pi content in layer a of the C3.

c) Comparisons between raced and non-raced horses

The Ca and Pi content was significantly greater in the raced compared to the non-raced horses in layers c and d of the C3 (refer to Figure 2.21).

d) Pattern of the Ca and Pi content throughout the depth of the bone

The pattern of the Ca and Pi content throughout layers a to d in both the raced and non-raced horses is shown in Figure 2.21. The pattern differs throughout the depth of the bone in the raced and non-raced; similar to the Cr, in the racehorses the content increases slightly from layer a to b, and subsequently decreases from layers b to c, from which point the content increases slightly. In the non-racehorses however, the content declines from layers a to c and then increases from layers c to d. The variation amongst the layers a to d is significant ($p<0.0001$).

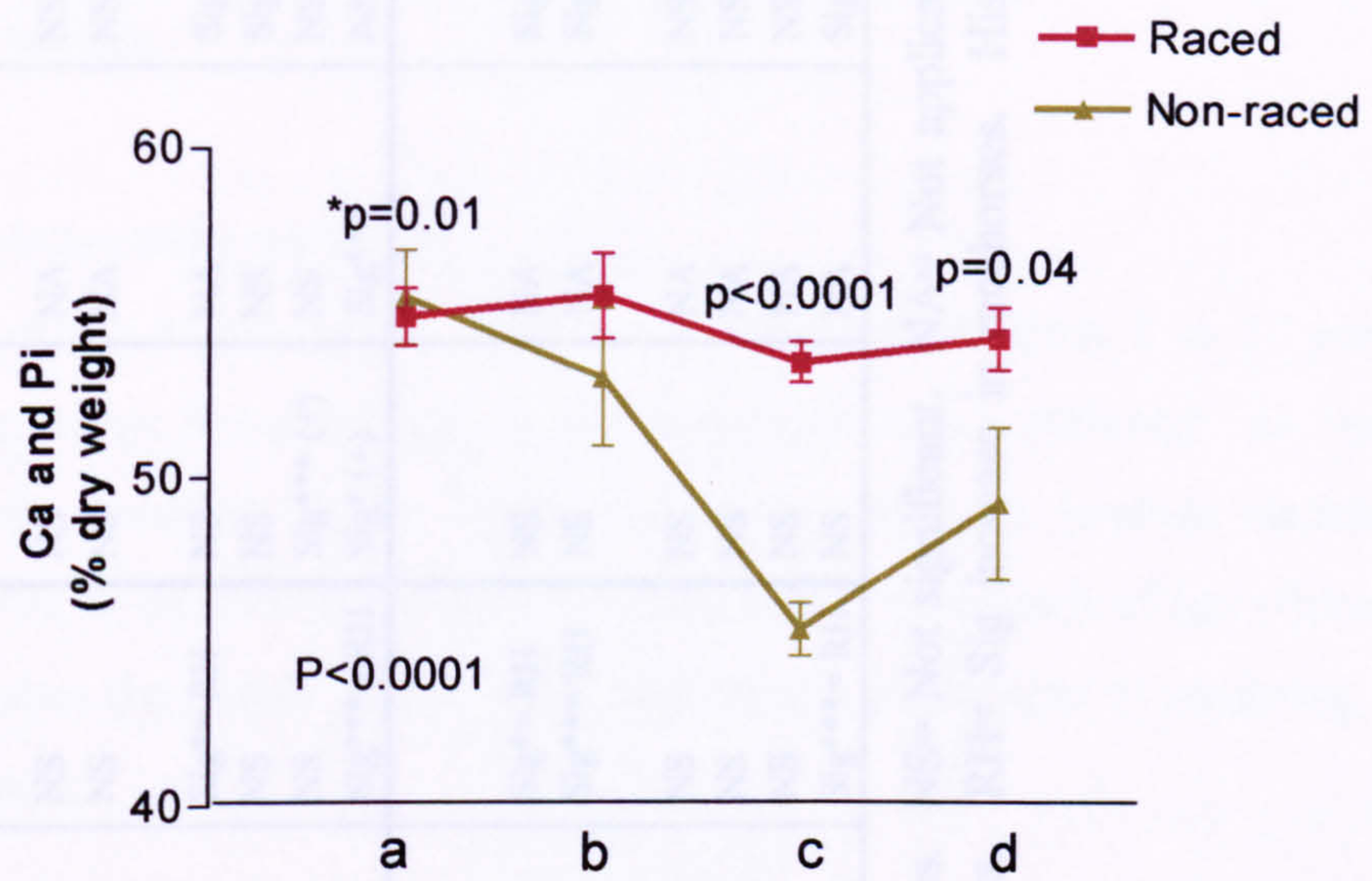


Figure 2.21: The Ca and Pi content throughout layers a to d of the C3. There was a significant difference between the raced and non-raced horses in layers c ($p<0.0001$) and d ($p=0.04$). The variation amongst the layers was significant ($p<0.0001$). Note: *p represents the p value given when there was a significant covariation with age.

	Layer a			Layer b			Layer c			Layer d		
	Correlation with Age	Covariation with Age	Significant Difference RH & NR	Correlation with Age	Covariation with Age	Significant Difference RH & NR	Correlation with Age	Covariation with Age	Significant Difference RH & NR	Correlation with Age	Covariation with Age	Significant Difference RH &NR
Cr												
BMD:												
Whole Bone	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
ROI	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
Histo:												
Bone Area	NS	NA	NS	NS	NA	NS	NS	NA	Sig*= RH	NS	NA	Sig***= RH
B.Pm/B.Tr	Sig*** (+)	NS	Sig***= NR	Sig** (+)	Sig**	NS	Sig* (+)	NS	NS	NS	NS	Sig*= NR
B.Pm/B.Ar	Sig** (+)	NS	Sig***= NR	Sig*** (+)	Sig*	NS	Sig*** (+)	Sig*	NS	Sig*** (+)	NS	NS
Ca and Pi:	NS	NA	NS	NS	NA	NS	NS	NA	Sig***= RH	Sig* (+)	Sig***	NS
C3												
BMD:												
Whole Bone	NS	NA	NS	NS	NA	NS	NS	NA	Sig*= RH	NS	NA	Sig*= RH
ROI	NS	NA	Sig*= RH	NS	NA	NS	NS	NA	Sig**= RH	NS	NA	Sig*= RH
Histo:												
Bone Area	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
B.Pm/B.Tr	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
B.Pm/B.Ar	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
Ca and Pi:	Sig* (+)	Sig*	NS	NS	NA	NS	NS	NA	Sig***= RH	NS	NA	Sig*= RH

Table 2.7: Summary of the mineral properties of the Cr and C3 layers a-d of raced and non-raced horses. NS= Not significant. NA= Not applicable. Sig= Significant results= *(P<0.05), **(P<0.01), ***(P<0.001). NR= Sig increase in non-racehorses. RH= Sig increase in racehorses. Histo= Histomorphometry. (-)= Negative correlation with age. (+)= Positive correlation with age.

2.4 DISCUSSION

Relationships with Age

The ages of the horses in this study varied considerably from 3 to 22 years, with the widest age range being evident in the non-racehorses. Although no very immature horses were included in this study, the horse does not become skeletally mature, characterised by closure of the growth plate, until 3 to 4 years of age (Price, Jackson et al. 1995) thus the horses in this study represent a population of maturing, mature and ageing horses.

A relationship with age and the histomorphometric bone perimeter measurements was evident in the Cr of the horses in this study, suggesting that with age there is possibly an increase in bone resorption resulting in increased bone porosity. This finding is similar to that previously reported in the navicular bone of horses aged from 2 years to greater than 12 years old (unfortunately the age of the oldest horses was not given) (Gabrie, Detilleux et al. 1999). In that study, the histomorphometrical data demonstrated that the cortical bone volume decreased with age, whereas cancellous (trabecular) bone volume and, in particular, the marrow spaces increased (Gabrie, Detilleux et al. 1999).

Interestingly, a relationship with age and BMD was not observed in the Cr in this study. A decline in bone density with increased porosity would have been expected. This may however be attributable to the different sites within the bone from which the BMD and the histomorphometrical measurements were taken, providing evidence that different areas of the bone are subjected to varying degrees of loading during exercise.

Moreover, no such relationship with the histomorphometrical measurements or BMD with age were found to exist in the C3. Exercise has been previously reported in humans to prevent age-related loss in bone (Hurley and Roth 2000; Karlsson 2002; Karlsson 2002; Lucas, Lucas et al. 2003), which was also suggested in the study by Gabrie *et al.*, (1999). Although in most horses in that study the navicular cortical bone volume decreased and the cancellous bone volume increased with age, this was not apparent in the sporting horses. In those horses the opposite occurred; cortical bone volume

increased with age and the cancellous bone volume decreased. Perhaps this lack of age relationship with bone perimeter and density in the C3 is due to the exercise undertaken by the horses in this study, since even the non-racehorses had been undertaking some form of exercise prior to euthanasia. Why this does not occur in the Cr may be due to the increased load absorbed by the C3 during exercise, compared to the Cr, and as such the C3 responds more rapidly to increased loading.

Right and Left Differences

Relatively few significant differences were observed between the right and left BMD and total Ca and Pi measurements, suggesting an even distribution of load between the right and left Cr and C3. Interestingly, any differences found were more frequent in the non-racehorses compared to the racehorses.

Young *et al.*, (1991), also found that the stiffness and radiographic density did not differ between the right and left C3 of the racehorses, even though these horses had been race-trained in a consistent anti-clockwise direction (Young, Richardson et al. 1991). The results from this study and those of Young *et al.*, (1991), are in accordance with data derived from *in vitro* studies that have demonstrated uniformly distributed intra-articular contact pressures across the dorsal proximal articular surface of the C3 when the midcarpal joint is fully extended (Palmer, Bertone et al. 1994).

The difference observed between left and right in the non-racehorses in this study may be a chance finding or may suggest that some horses preferentially lead with one forelimb more than the other. It may further reflect the exercise undertaken by these 'pleasure' horses, most probably ménage schooling whereby horses are exercised in small arenas, which due to rider preference is often in one direction more so than the other. In addition, in humans BMD in the carpus has also been shown to be increased in one hand comparable to the other, reflecting the preferential use of one hand over the other (Taaffe, Lewis et al. 1994; Tsuji, Tsunoda et al. 1995).

The Effects of Exercise

Bone Mineral Density

The increase in the BMD evident in the racehorses, particularly in the C3, suggests that racing and race-training is inducing osseous bone formation, possibly in an attempt to help strengthen the bone. These results are similar to that previously documented in treadmill exercised horses (Firth, Delahunt et al. 1999; Firth, Goodship et al. 1999); Firth *et al.*, (1999), showed that a high-intensity treadmill exercise protocol resulted in an increased BMD, quantified using DEXA, in the Cr and C3 of exercised TB horses compared to non-exercised controls (Firth, Delahunt et al. 1999). Similarly, Riggs *et al.*, (1999) showed that bone density (obtained via CT) was greater in the cortical and trabecular regions of the MCIII of treadmill exercised horses compared to non-treadmill exercised controls (Riggs and Boyde 1999).

The greatest difference in BMD observed between the two groups in this study was in the trabecular layers c and d of the C3. This is similar to the results presented in the treadmill study by Firth *et al.*, (1999), in which it was shown that the BMD in one of the subregions, the central third of the dorsal aspect, approximately equivalent to layer c in this study, was 28% greater in the exercised than the control horses (Firth, Delahunt et al. 1999). This suggests that even within trabecular bone, osseous tissue is highly responsive to the increased mechanical load, resulting in a net increase in bone formation in the C3 of racehorses in this study. However, the reason for this osteoinductive response of the trabecular bone is unclear, but may result from the increased accumulation of microfractures, which has recently been demonstrated in the C3 (Kawcak, Norrdin et al. 1998) and the distal MCIII (Reilly, Currey et al. 1997) subchondral bone of horses exercised at high speed on a treadmill.

Overall, the BMD in the Cr and C3 of both the raced and non-raced horses was found to decline with the depth of the bone i.e. from layers a to d, with the deeper layers having less volumetric BMD (g/cm^3) than the layers closer to the articular cartilage. This phenomenon was also documented in the study by Firth *et al.*, (1999), in which the BMD in the most dorsoproximal region of the C3 was greatest compared to the other more distal (and palmar) subregions (Firth, Delahunt et al. 1999). This presumably reflects the

response of the cortical bone to the mechanical loading, even the light to medium intensity exercise undertaken by the non-racehorses, since it is the cortical region directly underlying the articular cartilage, which is absorbing the most load during physical exercise, and hence which may be becoming more dense in an attempt to increase bone strength.

The increased BMD observed in the C3 of the racehorses would presumably result in a concomitant increase in subchondral bone stiffness, a response which has been documented by Young *et al.*, (1991) in the C3 of racehorses (Young, Richardson et al. 1991) and in the cortical and trabecular bone of OA human specimens (Li and Aspden 1997). Unfortunately, the mechanical properties were not obtained on the Cr and C3 of the specimens in this study, but if this response did apply to these specimens, this increase in bone stiffening may alter the share of the compressive load absorbed by the bone and cartilage during high-intensity exercise, possibly having deleterious results on the cartilage.

Overall, the difference in the volumetric BMD between the raced and non-raced horses in this study are not as marked as those documented by other studies which have quantified the effects of exercise on BMD in horses (Barneveld and van Weeren 1999; Cornelissen, van Weeren et al. 1999; Firth, Delahunt et al. 1999; Firth, Goodship et al. 1999; Firth, van Weeren et al. 1999; Riggs and Boyde 1999). Specifically within this study no differences in the BMD of the Cr were found to exist between the two groups. These results may reflect the nature of the work being undertaken by the non-racehorses, primarily light to medium intensity exercise, and further emphasise the acute responsiveness of bone to any form of physical exercise.

Histomorphometry

The bone area was found to be increased and, conversely the bone perimeter decreased, in the cortical and trabecular layers of the Cr in the raced compared to the non-raced horses. The increase in bone area and decrease in bone perimeter further suggests that racing and race-training is stimulating bone formation and inhibiting bone resorption so to maintain bone mass, again, in an attempt to strengthen the bone. Increased subchondral bone thickness, obtained via histomorphometrical analysis, has been

previously documented to occur in the carpal bones, particularly in the radial carpal bone, third carpal medial facet and the third carpal lateral facet, of treadmill exercised two year old horses compared to non-treadmill exercised controls (Murray, Vedi et al. 2001). McCarthy *et al.*, (1992), have additionally shown an increase in bone area of the MCIII of treadmill exercised yearlings compared to controls (McCarthy and Jeffcott 1992). Similarly, an increase in bone area has been documented in the CTB of racing Greyhounds (Johnson, Muir et al. 2000).

As for the BMD data, in both the raced and non-raced horses the bone area declined and conversely the bone perimeter increased, particularly within the racehorses, with the depth of the bone, the deeper layers having less bone per unit of tissue. This is consistent with an opening up of the bone, the bone becoming more porous and more 'trabecular' with depth from the articular surface.

Unfortunately, the presence of osteoid could not be determined in the specimens used within this study. However, an increased osteoid perimeter (used to represent the proportion of bone surface occupied by osteoid) has been previously shown to be associated with high-intensity exercise in the carpus of horses (Murray, Vedi et al. 2001), suggesting that exercise increases bone matrix production within the midcarpal joint.

One surprising finding within this study is that the increased bone area was only observed within the Cr of the racehorses, whereas the increased BMD was only observed in the C3 of the racehorses. This might suggest that the Cr and C3 respond differently to mechanical loading.

Total Calcium and Inorganic Phosphate

The total Ca and Pi content was generally greater in layers a to d of both the Cr and C3 of the racehorses and consistent with the BMD data, this increase was found to be greatest in layers c and d of the C3. In a recent study by Brama *et al.*, (2001) on the effect of training on the subchondral bone of five month old Dutch Warmblood foals, the calcium content was found to be increased in the right MCP joint of trained compared to untrained foals (Brama, Bank et al. 2001). This increase in total Ca and Pi with high-

intensity exercise suggests further that with increased loading, bone responds by attempting to become stronger.

Summary

Previous studies have shown high-intensity treadmill exercise to increase osseous bone formation but this is the first study to document such a response within the midcarpal bones of conventionally trained racehorses compared to non-racehorses of the same breed.

The racing associated osseous bone formation within the cortical and trabecular layers of the Cr and C3 is possibly a response to help strengthen the bone. The increased thickening/ density of the bone, and its presumed increased stiffness, may however, reduce the compliance and shock absorbing capacity of the bone. This alteration in mechanical loading may have consequences for other joint tissues, especially cartilage in the long term.

CHAPTER THREE

Bone Collagen Remodelling in the Midcarpal Joint of the TB Horse – Relationships to Exercise and Age

3.1 BONE COLLAGEN METABOLISM

3.1.1 General Introduction

Candidate biochemical markers of bone collagen metabolism including the carboxyterminal propeptide of type I collagen (PICP) and the pyridinoline cross-linked telopeptide of type I collagen (ICTP), have been quantified for many years in the serum and urine of various species, including the horse, to aid diagnosis of skeletal diseases and assess bone quality (Price, Jackson et al. 1995; Price, Jackson et al. 1995; Hiney, Potter et al. 2000; Price, Jackson et al. 2001; Vervuert, Coenen et al. 2002). Furthermore, it has been shown that many of these biomarkers are influenced by age (Price, Jackson et al. 1995) and by recent exercise (Roos, Dahlberg et al. 1995). However, body fluid biomarkers fail to provide detailed information on the fundamental changes occurring in the bone. As previously outlined, the effects of exercise, and to a lesser extent age, on measures of bone density have been extensively documented in the horse. However, the effect and influence of exercise and age on bone collagen metabolism has to date received scant attention, and since a tissue with a high turnover (or remodelling) of collagen is often weaker and hence mechanically compromised (Mansell and Bailey 1998; Anderson-MacKenzie, Billingham et al. 1999; Wang, Bank et al. 2001), knowledge of the collagenous matrix of the tissue is crucial.

Studies by Brama *et al.*, (2001, 2002) have shown in the MCP joint of 5-month-old foals subjected to a daily exercise programme consisting of an increasing number of gallop sprints to have an altered collagen composition, primarily, increased mature cross-link (the immature cross-link content was not quantified) and hydroxylysine content, at the site most loaded during high speed exercise compared to non-exercised foals (Brama, Bank et al. 2001; Brama, TeKoppele et al. 2002). However, these studies only assessed

the collagenous matrix in the subchondral bone of the 5-month-old foals and hence to date no information exists on the effects of exercise on the organic matrix of maturing and mature horses. Kiiskinen *et al.*, (1978) and Suominen *et al.*, (1980) have also demonstrated an altered collagenous matrix with exercise in mice, concluding that prolonged physical activity affected the organic matrix of long bones. Again these studies were undertaken on skeletally immature animals (Kiiskinen and Heikkinen 1978; Suominen, Kiiskinen et al. 1980). The collagenous matrix of trabecular bone has also been shown to be modified in the femoral necks of treadmill-exercised rats compared to non-exercised controls (Salem, Zernicke et al. 1993), thus suggesting that modifications to the organic matrix of bone are not solely occurring in the subchondral or cortical bone.

The aim of this study was to investigate the biochemical and thermal properties of bone collagen in the cortical and trabecular regions of the Cr and C3 of raced and non-raced horses, to provide information on the effect of racing and race-training on the collagenous matrix of bone formed during exercise-induced remodelling. Relationships with age were also evaluated in this study.

3.1.2 Bone Collagen Synthesis

3.1.2.1 Introduction

3.1.2.1.1 Collagen Cross-Links

It is well established that the collagen fibre is stabilised by a series of covalent cross-links between the collagen molecules making up the fibre (Eyre, Paz et al. 1984) and hence the contribution of these cross-links to provide tensile strength to the tissue is considerable. The absence or reduction in collagen cross-links in bone can have devastating effects on the structural and material properties of the bone (Oxlund, Barckman et al. 1995).

Bone collagen is predominantly cross-linked by the immature ketoimine cross-link, hydroxylysino-keto-norleucine (HLKNL), due to a higher level of lysyl hydroxylation, and to a lesser extent the aldimine cross-link, dehydro-hydroxylysinonorleucine (deH-

HLNL) (Kielty, Hopkinson et al. 1993). During maturation these immature cross-links are converted to the stable trivalent cross-links, hydroxylysyl-pyridinoline (HL-Pyr), lysyl-pyridinoline (Lys-Pyr), and the putative cross-link pyrrole; Lys-Pyr being the most commonly found mature cross-link in bone (refer to Chapter One) (Knott and Bailey 1998). Thus with maturation the composition of the bone collagen cross-link changes, the main change being a decrease of the immature cross-links paralleled with an increase in the mature cross-links (Bailey, Paul et al. 1998).

High levels of immature cross-links, and conversely low levels of mature, cross-links are characteristic of a bone with elevated collagen remodelling. An accurate determination therefore of the ratio of the immature to mature cross-links provides a valuable indication of the degree of collagen turnover in bone.

3.1.2.1.2 Carboxyl-terminal Propeptide of Type I Collagen (PICP)

During the conversion of type I procollagen to type I tropocollagen, the C and N terminal propeptides are cleaved by specific extracellular tissue endopeptidases, releasing the propeptide into the ECM. These stable, soluble propeptides are present at a stoichiometric ratio of 1:1 to each collagen molecule synthesised and as such are excellent markers of collagen synthesis.

The extension that is cleaved from the carboxyl-terminal, known as the carboxyl-terminal propeptide of type I collagen (PICP) has been measured in serum for several years as a marker of bone collagen formation (Price, Jackson et al. 1995; Hiney, Potter et al. 2000). PICP has been quantified in horse sera using a commercially available radioimmunoassay (RIA) (Orion Diagnostica, Finland) (Price, Jackson et al. 1995; Price, Jackson et al. 1995; Hiney, Potter et al. 2000; Price, Jackson et al. 2001). However, to date PICP has not been quantified in equine bone extract.

Serum levels of PICP have been shown to correlate negatively with age in normal horses, (Price, Jackson et al. 1995; Price, Jackson et al. 1995; Price 1998). The study by Price *et al.* (1995) analysed PICP levels from the serum of 60 horses ranging from 3 months to 20 years of age. As expected, levels were highest in foals at a time of very rapid bone

modelling and were lowest in adult animals where bone modelling is decreased (Price, Jackson et al. 1995).

3.1.2.1.3 Bone-Specific Alkaline Phosphatase (BAP)

Although BAP, an isoenzyme of alkaline phosphatase, is not a marker of collagen synthesis, its secretion by osteoblasts during bone formation makes BAP a widely used non-collagenous marker of bone formation (Price, Jackson et al. 1995; Price, Jackson et al. 1995; Price 1998; Shibata, Ohsawa et al. 2003). It has been shown by Henson *et al.*, (1995) that in horses, BAP activity is localised on the surface membrane of chondrocytes with concentrated activity on the matrix vesicles of the growth plate (the putative site of mineralisation) thus supporting the use of BAP as a specific marker of bone formation in horses (Henson, Davies et al. 1995). BAP has long been associated with the mineralisation of bone (Robison 1923; Golub 1996), although, the role of BAP in this process is still debated. BAP has been suggested to be necessary for the formation of hydroxyapatite molecules by liberating the necessary phosphate molecules (Golub 1996).

As with most biochemical markers, BAP activity has been shown to decrease with age as bone modelling declines. An inverse correlation between age and serum BAP and total ALP with a marked decrease of activity within the first three weeks of life has been documented in 'normal' horses (Lepage, Marcoux et al. 1990; Lepage, DesCoteaux et al. 1991; Hank, Hoffmann et al. 1993; Price, Jackson et al. 1995).

3.1.2.2 Materials and Methods

Details of the materials and solutions used in the following methods are listed in Appendices Four and Five respectively.

3.1.2.2.1 Equine Samples

The pulverised bone obtained subsequent to BMD and mineral quantification (refer to Chapter 2 section 2.2.3) was used in the following analyses. The left and right Cr and C3 of 9 racehorses and 11 non-racehorses were quantified (refer to Table 3.1).

Age and Gender

The ages and gender of the horses used in the following analyses are shown in Table 3.1.

Raced	Age	Gender	Non-raced	Age	Gender
R1	7	G	**1**	17	M
R2	7	M	**2**	NA	G
R3	8	G	**3**	15	G
R4	4	C	**4**	22	G
R5	6	C	**5**	9	M
R6	7	G	**6**	12	G
R7	7	C	**7**	10	M
R8	7	C	**8**	11	M
R9	6	C	**9**	8	M
			10	NA	G
			11	3	G

Table 3.1: The age (years) and gender of the horses. (C= Colt, G= Gelding (neutered male), M=mare, NA= Not Known).

3.1.2.2.2 Collagen Cross-link Quantification

Tissue Preparation

A proportion of the pulverised bone was decalcified in 0.5M EDTA (tetrasodium salt) pH 7.5 for 2 days (at a concentration of 10mg (wet weight)/5ml EDTA) with a change of EDTA after the first day, and subsequently washed in dH_2O (x2) to remove the EDTA.

Borohydride Reduction and Tissue Hydrolysis

The cross-links were quantified as described by Sims and Bailey (Sims and Bailey 1992). The demineralised bone was suspended in 1.5ml phosphate buffered saline (PBS) followed by 30µl 1mg/ml potassium borohydride in 0.001M NaOH to stabilise the immature cross-links (deH-HLNL to HLNL and HLKNL to diHLNL respectively). The reduction was stopped after 1hr by the addition of 250µl glacial acetic acid and the samples washed several times in distilled water before freeze-drying. The samples were weighed and then hydrolysed in 6N HCL (at a concentration of 5mg/ ml HCL), at 110°C overnight in a 6ml screw top polypropylene hydrolysis tube and then freeze-dried overnight.

Isolation and Preparation of Collagen Cross-Links

Collagen cross-links (and other amino acids) can be isolated from the hydrolysate mixture by way of a simple hydrophobic interaction using a CF-1 cellulose (Whatman, Kent, UK) chromatography mini-column.

In detail, the hydrolysate was resuspended in 600µl dH_2O. 100µl was used for hydroxyproline content analysis as described below. 500µl of glacial acetic acid was then added to the remaining 500µl and the mixture transferred from the hydrolysis tube to a 7ml plastic universal. The hydrolysis tube was then washed (x2) with 1ml butanol and decanted into the bijou, so that it contained 2ml butanol, 500µl hydrolysate solution and 500µl acetic acid. This mixture at a ratio of 4:1:1 is the 'organic phase' that enables the collagen cross-links to bind to the cellulose column. The CF1-cellulose column was prepared by suspending the CF1 powder in organic phase, this was subsequently allowed to settle then the excess poured away to remove any fine particles and fresh organic phase added. The resulting CF1 mixture was added to the mini-column with a column height of 8cm after settling the bed with 8ml of the organic phase. The 3ml hydrolysate/ organic phase sample was added to the mini-column, and the columns then washed with 16ml of the organic phase to remove the majority of the non-cross-linked amino acids. The remaining cross-linked amino acids were then eluted from the CF1-cellulose by washing with 8ml dH_2O and the effluent collected in labelled 25ml sterilin tubes and freeze-dried.

Quantification of Collagen Cross-Links

After freeze-drying, the samples were dissolved in 100µl of 0.01M HCL, vortexed, filtered through 0.2µ filters and then applied to an amino-acid analyser (Alpha Plus. Pharmacia, Loughborough, UK) configured for the separation of cross-linking amino acids using authenticated cross-links as standards. Quantification was by colour reaction with ninhydrin. The elution profile of the cross-link standards is illustrated in Figure 3.1.

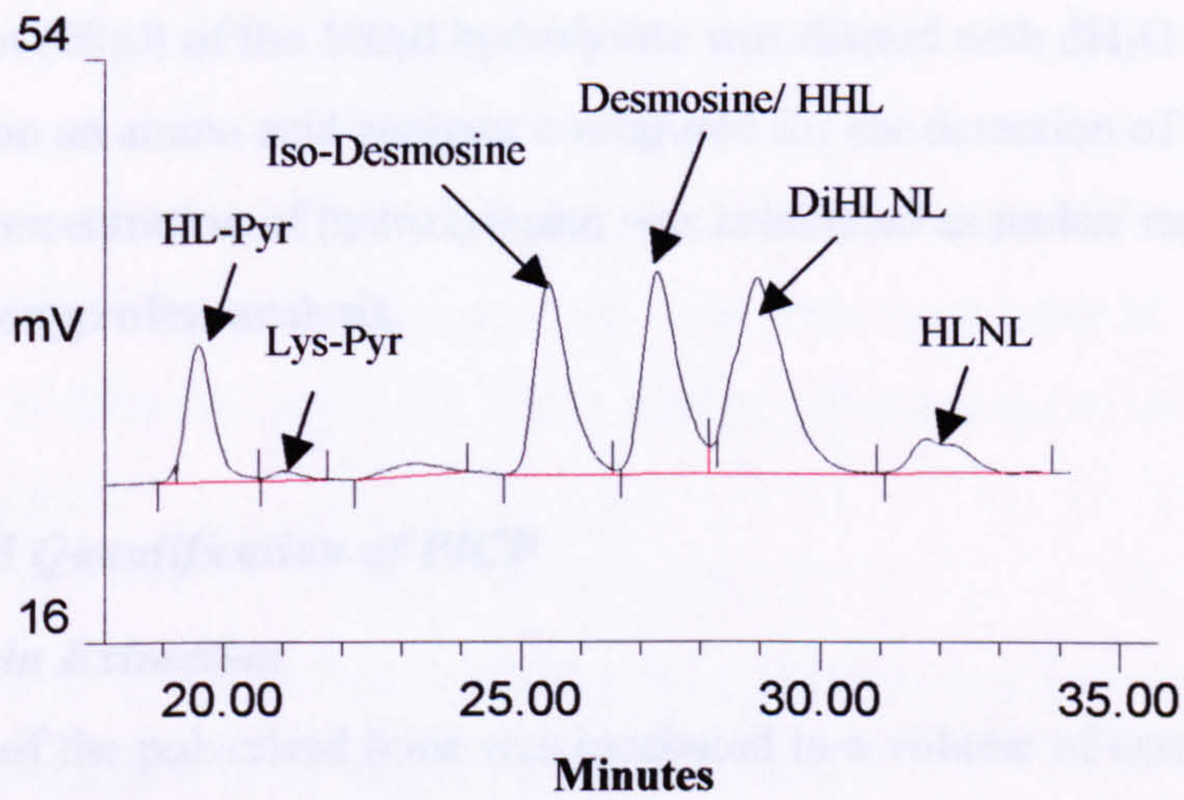


Figure 3.1: Typical elution profile of the collagen and elastin cross-link standards on the amino-acid analyser.

3.1.2.2.3 Hydroxyproline Content Quantification

To enable the expression of the cross-link results as moles cross-link/ mole collagen, the amount of collagen in each sample was quantified. The amino acid hydroxyproline is largely restricted to collagen and since the vast majority of bone collagen is type I, which is known to have a hydroxyproline content of 14%, the collagen content of bone was calculated from the quantification of hydroxyproline. The method is based on that described by Bannister and Burns (Bannister and Burns 1970). The free hydroxyproline, liberated during hydrolysis, is initially oxidised by Chloramine T. The resulting pyrrole is coupled to 4-dimethylaminobenzaldehyde to give a coloured product of an absorbance at 550nm. A small aliquot (5µl) from the 100µl hydrolysate obtained during cross-link quantification (refer to section 3.1.2.2.2) was diluted up to a volume of 1ml to bring the

concentration of hydroxyproline within the range of the analyser (0-5µg/ml) and analysed on a continuous flow auto analyser (Burkland Scientific, Uxbridge, UK). Standards were prepared from a solution of L-hydroxyproline in 0.01M HCL. The collagen content was then calculated from the hydroxyproline values, based on a hydroxyproline content of 14% per collagen molecule.

3.1.2.2.4 Amino Acid Analysis for Lysine Hydroxylation

A small aliquot (20µl) of the 100µl hydrolysate was diluted with dH_2O (100µl up to 1ml) and analysed on an amino acid analyser configured for the detection of hydroxylysine and lysine. The concentration of hydroxylysine was calculated as moles/ mole collagen based upon the hydroxyproline analysis.

3.1.2.2.5 Quantification of PICP

Soluble Protein Extraction

A proportion of the pulverised bone was incubated in a volume of extract buffer (20mM triethanolamine/ 0.1% Brij 35 (w/v)), relative to the dry weight (20µl/mg) at 4°C for 12-18 hours. The sample was centrifuged for 20 minutes at 7500G, the supernatant removed and aliquoted to minimise freeze-thawing. The aliquots were stored at -20°C until required.

Assay Quantification

In order to assess the cross-reactivity of the commercially available RIA (Orion Diagnostica, Finland) with PICP in equine bone extract, bone extract from one animal was serially diluted (dilution of x0.375) with extract buffer and a standard curve plotted.

PICP RIA

The Orion Diagnostica PICP RIA kit is based on the competitive radioimmunoassay technique. 100µl of raced and non-raced bone extract, standard and control (lyophilised, human serum) were pipetted into test tubes, to which 200µl of PICP ^{125}I reagent and 200µl of PICP antiserum were added. After 2hrs incubation 500µl of separation reagent (ready to use suspension of a second antibody covalently bound to solid particles with

bovine serum albumin (BSA)) was added and incubated for a further 30 minutes. The tubes were centrifuged, the supernatant removed and the amount of labelled antigen quantified. The amount of labelled PICP in the tube is inversely proportional to the amount of PICP in the sample, in that, the lower the percentage bound to unbound (%B/Bo) radioactivity the higher the concentration of PICP in the sample.

3.1.2.2.6 Quantification of Alkaline Phosphatase (ALP)

For total alkaline phosphatase quantification, 100µl aliquots of the soluble protein extract (refer to section 3.1.2.2.5) were analysed on an automated analyser, Konelab 30i (Thermo Clinical Labsystems, Finland). ALP catalyses the hydrolysis of p-nitrophenylphosphate, yielding the coloured compound p-nitrophenol. The formation of p-nitrophenol is followed at 405nm and is directly proportional to the concentration of ALP in the sample.

Since the levels of ALP have been quantified from bone extract the concentration of ALP in the results is assumed to be bone-specific ALP (BAP).

3.1.2.2.7 Statistical Analysis

Statistical analysis was performed using SPSS v11.5 (SPSS Inc. USA) and Graphpad Prism v3.0 (Graphpad Software Inc. USA), as detailed in Chapter Two section 2.2.1.5.

3.1.2.3 Results

3.1.2.3.1 Age

The ages of the horses in this study varied from 3 to 22 years (mean age 9; SEM 1.2) with the widest age range being evident in the non-raced horses.

- Raced: Mean age= 6 years; SEM= 0.3.
- Non-raced: Mean age= 11 years; SEM= 1.8.

3.1.2.3.2 Gender

a) Total Mature Cross-links (Cr layers a and d)

There was no significant difference in the total mature cross-links in layer a (p=0.48) of the Cr between males (0.24+/-0.02) and females (0.28+/-0.06) and layer d (p=0.27) between males (0.20+/-0.04) and females (0.13+/-0.01).

b) BAP (C3 layers b and c)

There was additionally no significant difference in the BAP content in layer b (p=0.17) of the C3 between males (53.39+/-10.43) and females (28.92+/-9.6) and c (p=0.23) between males (43.18+/-8.47) and females (21.83+/-4.08).

3.1.2.3.3 Collagen Cross-links

The cross-link elution profile for equine bone is illustrated in Figure 3.2. The cross-link types were the same in both the cortical and trabecular layers (layers a-d) of bone.

Within this thesis the immature cross-links will be referred to in the reduced form, i.e. dihydroxy-lysinonorleucine (diHLNL) and hydroxylysinonorleucine (HLNL – assumed to be in the keto- form).

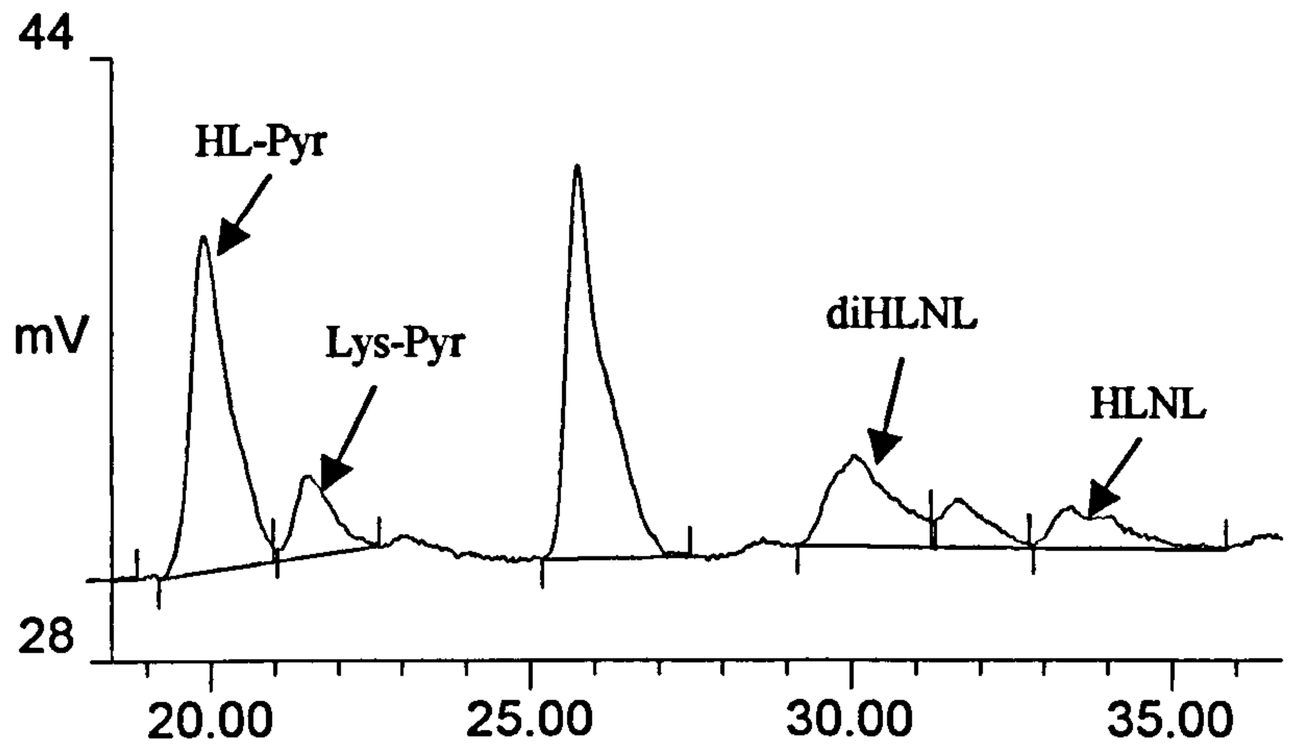


Figure 3.2: The elution profile of the cross-links in equine cortical bone (layer a).

1) Cr:

a) Right and left differences

i) diHLNL and HLNL (Immature)

There was no significant difference in the diHLNL and HLNL cross-link content between the right and left Cr in either the raced or non-raced horses layers a to d (see Appendix Three).

ii) HL-Pyr and Lys-Pyr (Mature)

There was a significant difference in the HL-Pyr content between the right (0.19+/-0.01) and left (0.17+/-0.01) layer b ($p=0.04$) of the non-raced horses. However, there were no other significant differences between the right and left HL-Pyr and Lys-Pyr content for either raced or non-raced horses layers a to d (see Appendix Three).

b) Correlation and covariation with age

Immature and Mature:

There was no significant correlation in the diHLNL and HLNL and HL-Pyr cross-link content with age throughout the depth of the Cr (layers a-d) of the TB horses (refer to Table 3.2). The ratios of diHLNL to HLNL negatively correlated with age in layers a, b and d (refer to Table 3.2) but there was only a significant covariation with age in layer b ($p=0.005$).

Lys-Pyr had a significant positive correlation with age in layers b and c (refer to Table 3.2). However, there was no significant covariation with age in layer b (p=0.46), but there was a significant covariation with age in layer c (p=0.04). The ratios of HL-Pyr to Lys-Pyr did not significantly correlate with age (refer to Table 3.2).

Layer	Linear (Pearson) Correlation (r^2)	P Value
diHLNL		
a	-0.007	0.73
b	-0.07	0.28
c	-0.03	0.45
d	-0.02	0.53
HLNL		
a	+0.12	0.15
b	-0.0007	0.91
c	+0.01	0.59
d	+0.03	0.44
diHLNL:HLNL		
a	-0.30	**0.01**
b	-0.23	**0.04**
c	-0.01	0.69
d	-0.33	**0.01**
HL-Pyr		
a	+0.17	0.08
b	+0.06	0.33
c	+0.16	0.09
d	+0.08	0.23
Lys-Pyr		
a	+0.17	0.08
b	+0.25	**0.04**
c	+0.41	**0.004**
d	0.16	0.09
HL-Pyr:Lys-Pyr		
a	-0.003	0.82
b	-0.05	0.37
c	-0.07	0.26
d	-0.03	0.48

Table 3.2: Correlation coefficients of the cross-link content and age in the Cr layers a-d.

c) Comparisons between raced and non-raced horses

Immature:

There was no significant difference in the diHLNL and HLNL cross-link content between the raced and non-raced horses in layers a to d of the Cr. However there was a significant difference in the diHLNL to HLNL ratios in layers b and d, being greater in the racehorses (refer to Table 3.3).

Layer	Raced	Non-raced	P Value
diHLNL			
a	0.12+/-0.01	0.15+/-0.03	0.65
b	0.65+/-0.24	0.36+/-0.07	0.43
c	0.51+/-0.18	0.23+/-0.05	0.45
d	0.33+/-0.09	0.16+/-0.03	0.29
HLNL			
a	0.07+/-0.01	0.11+/-0.02	0.41
b	0.29+/-0.09	0.34+/-0.05	0.63
c	0.51+/-0.18	0.23+/-0.05	0.17
d	0.21+/-0.05	0.16+/-0.03	0.51
diHLNL:HLNL			
a	1.83+/-0.18	1.66+/-0.36	0.71
b	1.82+/-0.30	0.93+/-0.09	**0.005**
c	0.73+/-0.23	0.46+/-0.09	0.28
d	1.52+/-0.15	1.06+/-0.14	**0.04**
HL-Pyr			
a	0.14+/-0.01	0.22+/-0.02	**0.03**
b	0.14+/-0.04	0.18+/-0.01	0.09
c	0.11+/-0.02	0.13+/-0.02	0.44
d	0.12+/-0.04	0.11+/-0.01	0.78
Lys-Pyr			
a	0.05+/-0.006	0.07+/-0.009	0.08
b	0.03+/-0.007	0.07+/-0.005	**0.001**
c	0.03+/-0.004	0.06+/-0.01	*0.04
d	0.05+/-0.01	0.06+/-0.009	0.66

Table 3.3: The mature and immature cross-link content in the Cr of raced and non-raced horses layers a to d (mean mole/mole collagen +/- SEM). Note: *p represents the p value given when there was a significant covariation with age.

Mature:

HL-Pyr content was significantly greater in the non-raced compared to the raced in the cortical layer a (p=0.03). There was a significant difference between the raced and non-raced horses in the Lys-Pyr content in layer b, being greatest in the non-raced (p=0.001). No other significant differences in the HL-Pyr and Lys-Pyr content between the two

groups of horses were observed (refer to Table 3.3). The ratios of HL-Pyr to Lys-Pyr in layers b to d were greater, but not significantly, in the raced compared to the non-raced horses (refer to Figure 3.3).

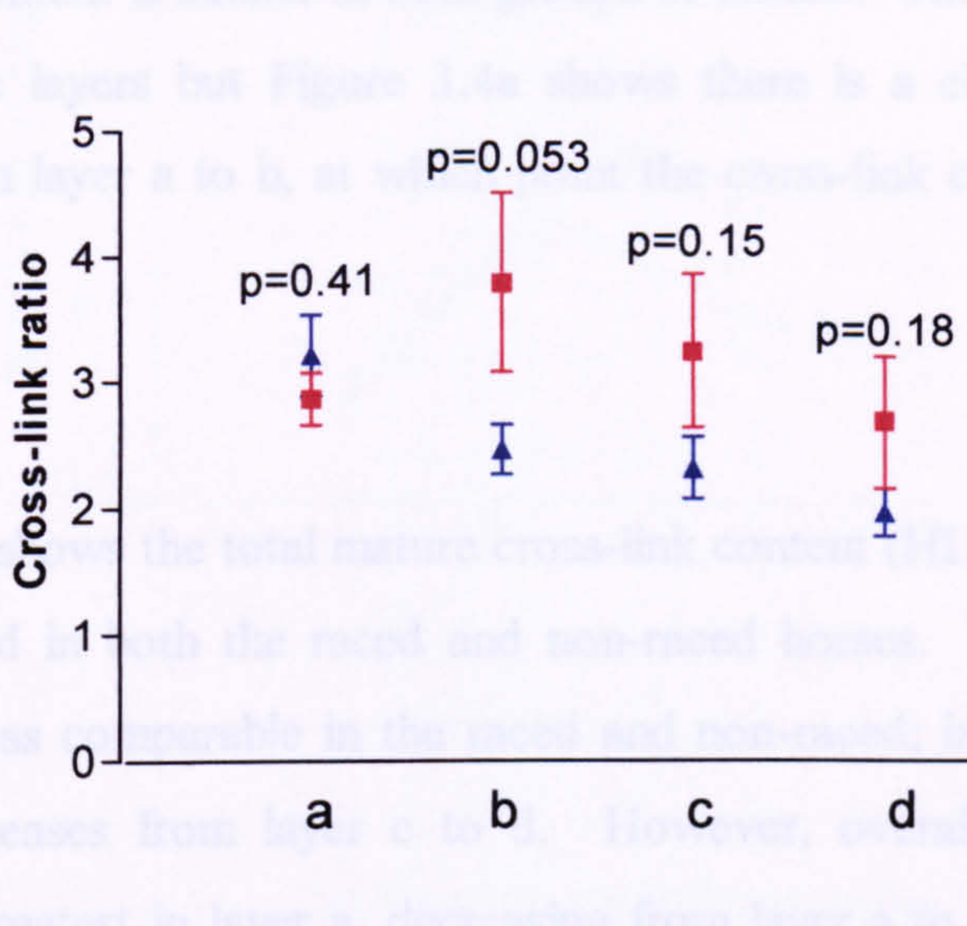


Figure 3.3: The HL-Pyr to Lys-Pyr cross-link ratio in layers a to d of the Cr.

Immature to mature ratio:

The ratio of immature (diHLNL plus HLNL) to mature (Lys-Pyr plus HL-Pyr) cross-links correlated with age in layer d (r^2= -0.22, p=0.04), however there was no significant covariation with age in this layer (p=0.21). The ratio was greatest, but not significantly, in all layers of the raced compared to the non-raced horses (refer to Table 3.4).

Layer	Raced	Non-raced	P Value
a	0.98+/-0.07	0.87+/-0.1	0.43
b	4.28+/-0.97	2.55+/-0.43	0.13
c	3.99+/-0.75	2.52+/-0.41	0.08
d	3.4+/-1.89	2.04+/-0.31	0.08

Table 3.4: The ratio of immature to mature cross-links in the Cr of raced and non-raced horses (mean mole/mole collagen +/- SEM).

d) Pattern of the cross-link content throughout the depth of the bone

Immature:

The total immature cross-link content (diHLNL plus HLNL) throughout layers a to d in both the raced and non-raced horses is shown in Figure 3.4a. The pattern of the collagen cross-link content is similar in both groups of horses. There was no significant variation amongst the layers but Figure 3.4a shows there is a clear increase in the cross-link content from layer a to b, at which point the cross-link content decreases linearly from layer b to d.

Mature:

Figure 3.4b shows the total mature cross-link content (HL-Pyr plus Lys-Pyr) throughout layers a to d in both the raced and non-raced horses. The pattern of the cross-link content is less comparable in the raced and non-raced; in the racehorses the cross-link content increases from layer c to d. However, overall, the total mature cross-link content is greatest in layer a, decreasing from layer a to d. Additionally, the variation amongst the layers is significant ($p=0.007$).

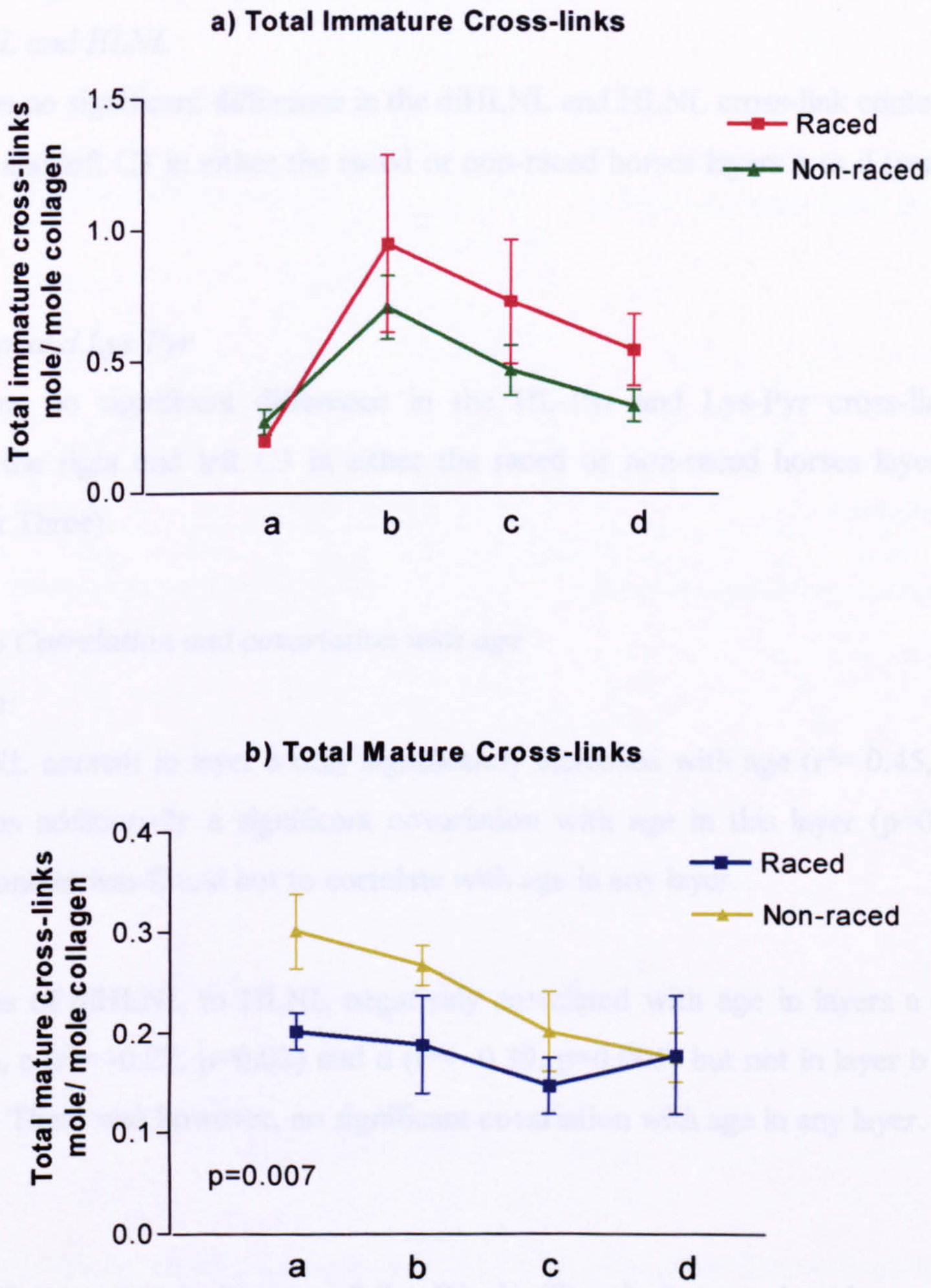


Figure 3.4: The total immature (a) and total mature (b) cross-link content in the various layers of the Cr. In graph b) the variation amongst the layers was significant (p=0.007).

2) C3:

a) Right and left differences

i) diHLNL and HLNL

There was no significant difference in the diHLNL and HLNL cross-link content between the right and left C3 in either the raced or non-raced horses layers a to d (see Appendix Three).

ii) HL-Pyr and Lys-Pyr

There was no significant difference in the HL-Pyr and Lys-Pyr cross-link content between the right and left C3 in either the raced or non-raced horses layers a-d (see Appendix Three).

b) Correlation and covariation with age

Immature:

The HLNL content in layer a only significantly increased with age (r^2= 0.45, p=0.002). There was additionally a significant covariation with age in this layer (p=0.01). The diHNL content was found not to correlate with age in any layer.

The ratios of diHLNL to HLNL negatively correlated with age in layers a (r^2= -0.36, p=0.007), c (r^2= -0.27, p=0.03) and d (r^2= -0.39, p=0.005) but not in layer b (r^2= -0.21, p=0.05). There was however, no significant covariation with age in any layer.

Mature:

The HL-Pyr content in layer a of the C3 significantly increased with age (r^2= 0.32, p=0.01), however, there was no significant covariation with age (p=0.42).

The Lys-Pyr content significantly increased with age in layers a, b and c (refer to Figure 3.5) but not layer d (r^2= 0.21, p=0.06). The Lys-Pyr cross-link content had no significant covariation with age in layer b (p=0.09) or layer c (p=0.87), but there was a significant covariation in layer a (p=0.007).

The HL-Pyr to Lys-Pyr ratio did not significantly correlate with age in any layer; layer a (r^2= -0.07, p=0.27), b (r^2= -0.10, p=0.19), c (r^2= -0.07, p=0.28) and d (r^2= -0.14, p=0.13).

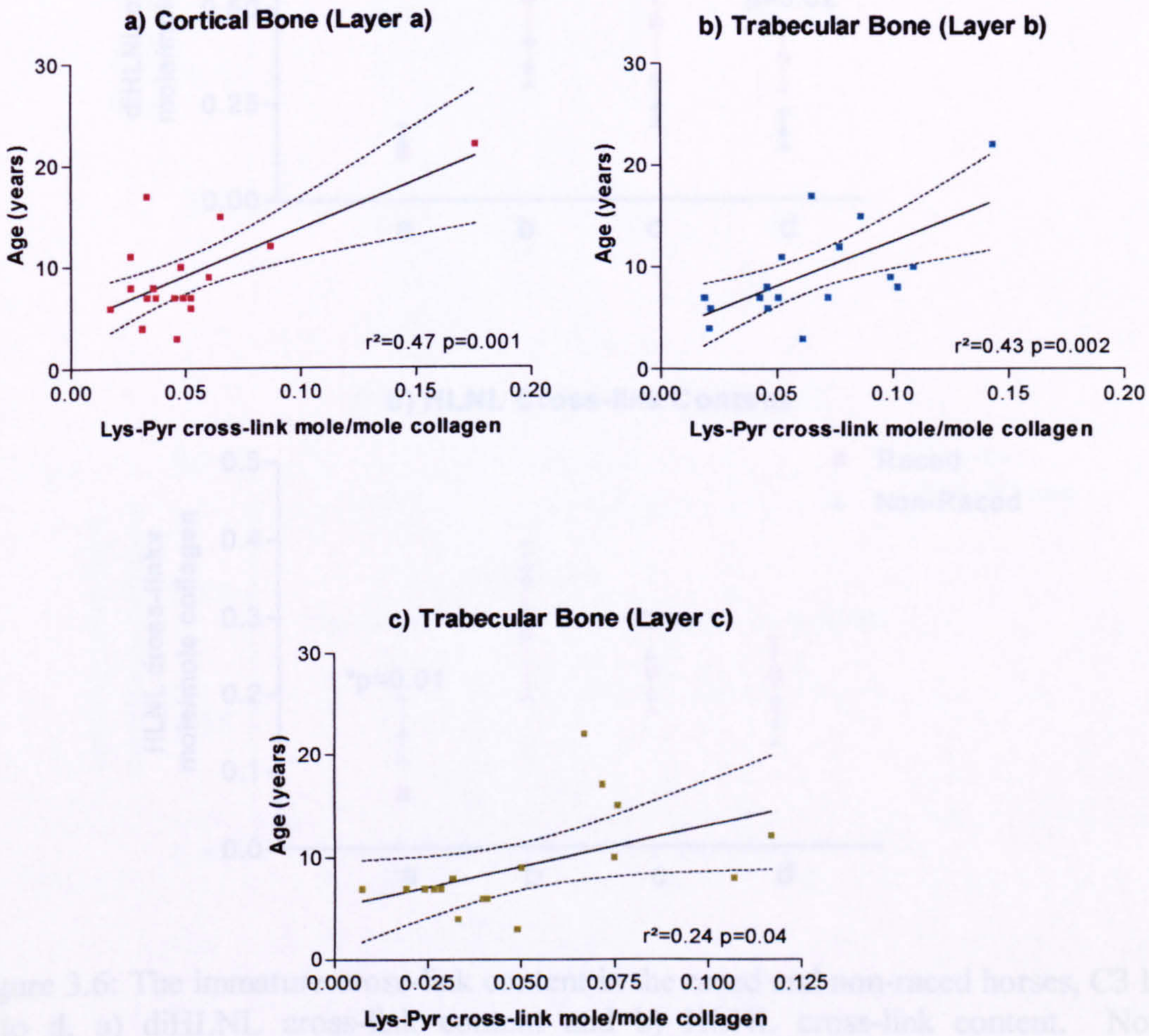


Figure 3.5: The correlation between age and the Lys-Pyr cross-link content in a) layer a, b) layer b and c) layer c of the C3.

c) Comparisons between raced and non-raced horses

Immature:

The levels of diHLNL were significantly higher in the racehorses compared to the non-raced in layer d (p=0.02) (refer to Figure 3.6a). There was no significant difference in the HLNL content between the raced and non-raced horses in any layer (refer to Figure 3.6b).

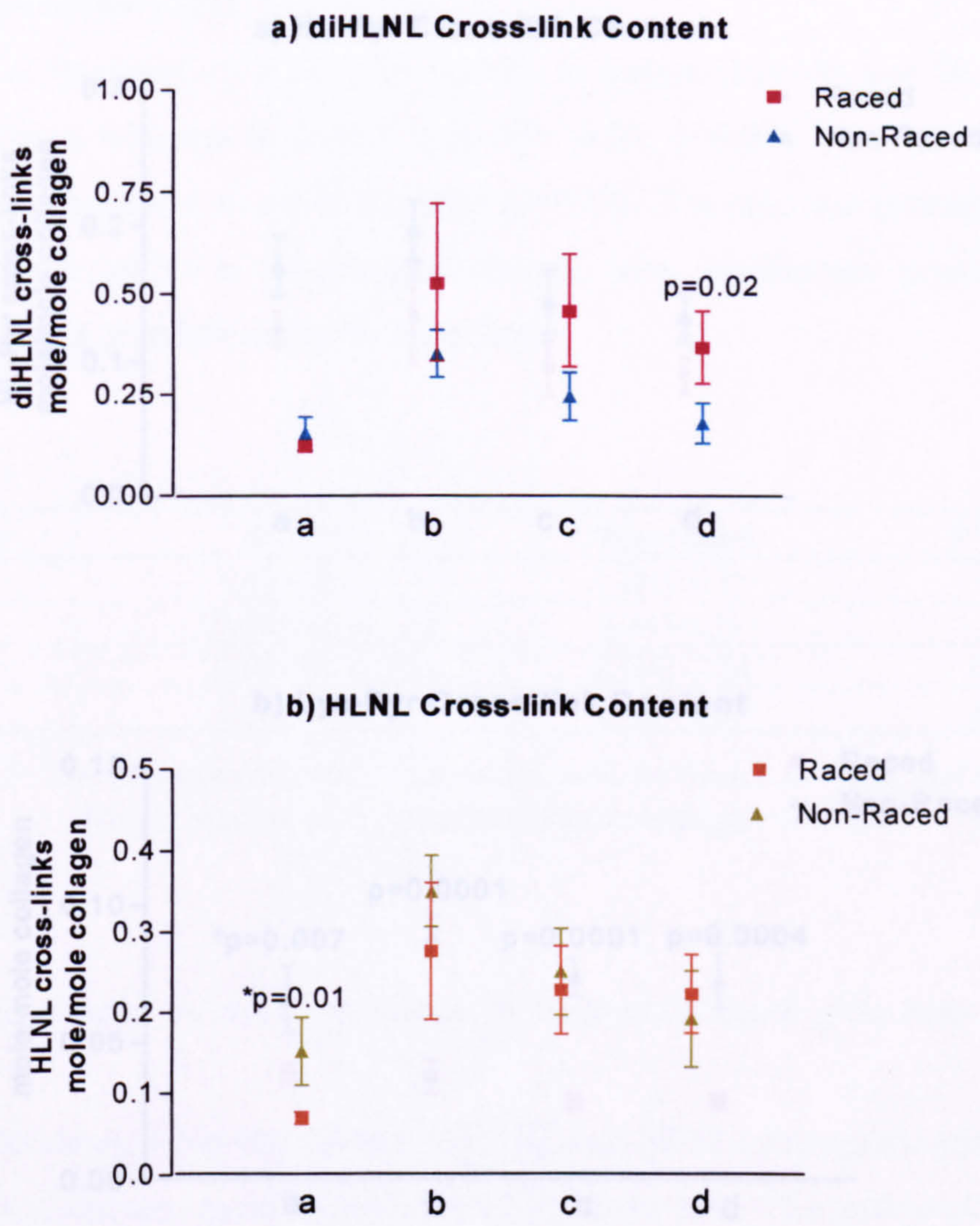


Figure 3.6: The immature cross-link content in the raced and non-raced horses, C3 layers a to d. a) diHLNL cross-link content and b) HLNL cross-link content. Note:*p represents the p value given when there was a significant covariation with age.

Mature:

There was no significant difference in the HL-Pyr cross-link content between the raced and non-raced horses (refer to Figure 3.7a). The Lys-Pyr content was significantly higher in the non-raced compared to the raced in layers b, c and d (refer to Figure 3.7b).

The ratio of HL-Pyr to Lys-Pyr was significantly higher in the raced than the non-raced horses in layers c and d (refer to Figure 3.7c).

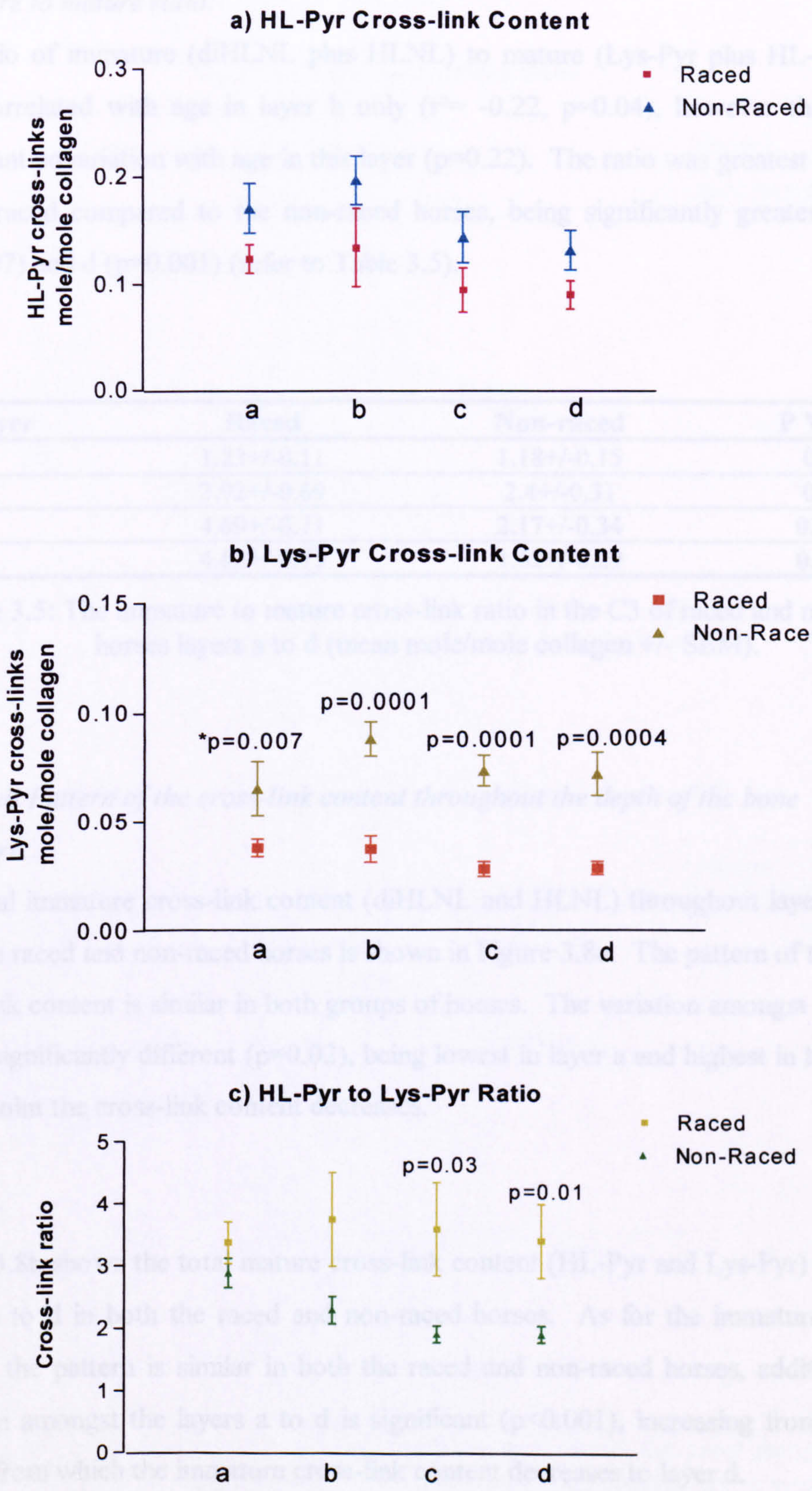


Figure 3.7: Mature cross-link content in the raced and non-raced horses, layers a to d. a) HL-Pyr cross-links, b) Lys-Pyr cross-links and c) HL-Pyr to Lys-Pyr ratio. Note: *p represents the p value given when there was a significant covariation with age.

Immature to mature ratio:

The ratio of immature (diHLNL plus HLNL) to mature (Lys-Pyr plus HL-Pyr) cross-links correlated with age in layer b only (r^2= -0.22, p=0.04), however there was no significant covariation with age in this layer (p=0.22). The ratio was greatest in all layers of the raced compared to the non-raced horses, being significantly greater in layer c (p=0.007) and d (p=0.001) (refer to Table 3.5).

Layer	Raced	Non-raced	P Value
a	1.23+/-0.11	1.18+/-0.15	0.78
b	3.92+/-0.69	2.4+/-0.31	0.07
c	4.69+/-0.71	2.17+/-0.34	**0.007**
d	4.68+/-0.75	1.62+/-0.29	**0.001**

Table 3.5: The immature to mature cross-link ratio in the C3 of raced and non-raced horses layers a to d (mean mole/mole collagen +/- SEM).

d) Pattern of the cross-link content throughout the depth of the bone

Immature:

The total immature cross-link content (diHLNL and HLNL) throughout layers a to d in both the raced and non-raced horses is shown in Figure 3.8a. The pattern of the collagen cross-link content is similar in both groups of horses. The variation amongst the layers a to d is significantly different (p=0.02), being lowest in layer a and highest in layer b from which point the cross-link content decreases.

Mature:

Figure 3.8b shows the total mature cross-link content (HL-Pyr and Lys-Pyr) throughout layers a to d in both the raced and non-raced horses. As for the immature cross-link content the pattern is similar in both the raced and non-raced horses, additionally, the variation amongst the layers a to d is significant (p<0.001), increasing from layer a to layer b from which the immature cross-link content decreases to layer d.

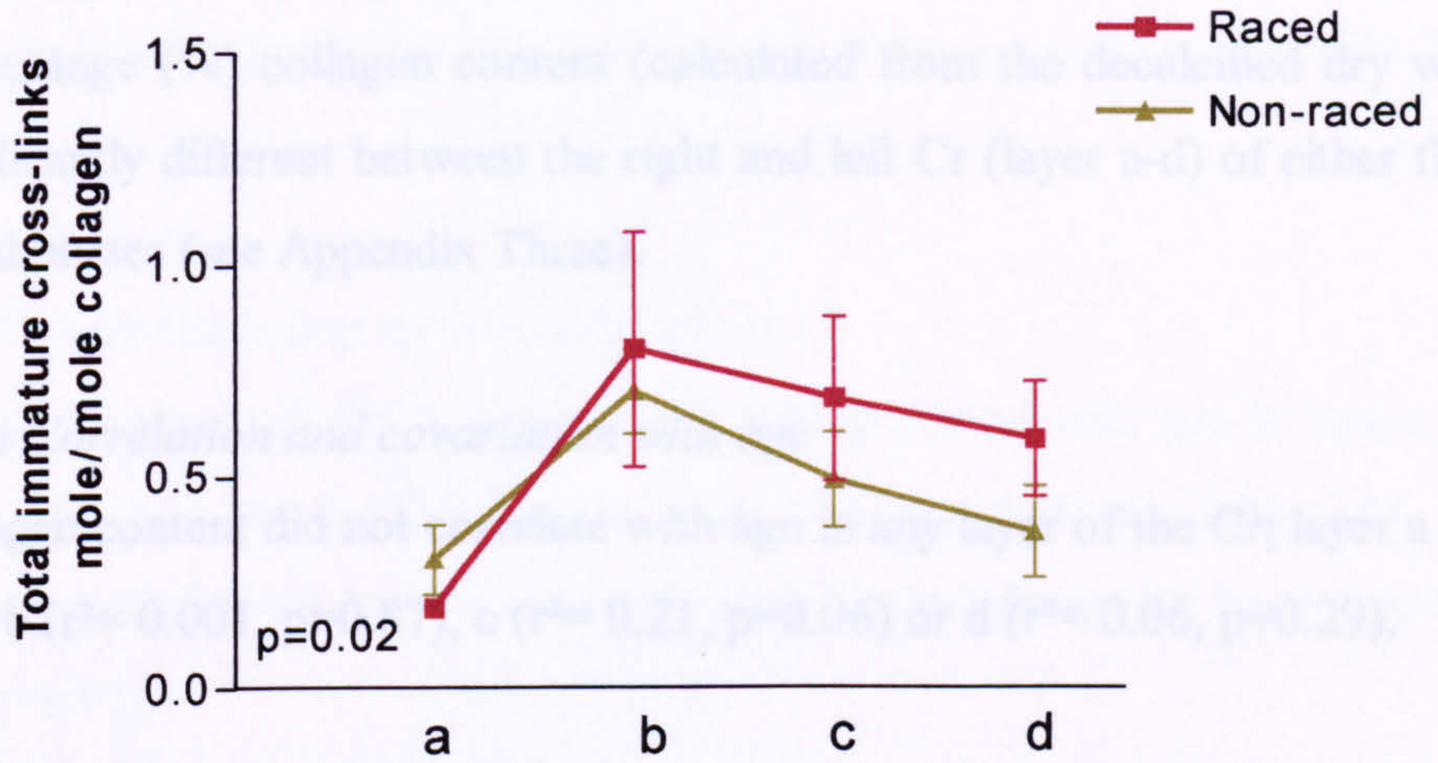


Figure 3.8: a) The total immature cross-link content and b) the total mature cross-link content in the various layers of the C3. The variation amongst the layers was significant; a) $p=0.02$ and b) $p<0.001$.

3.1.2.3.4 Collagen Content

1) Cr:

a) Right and left differences

The percentage (%) collagen content (calculated from the decalcified dry weight) was not significantly different between the right and left Cr (layer a-d) of either the raced or non-raced horses (see Appendix Three).

b) Correlation and covariation with age

The collagen content did not correlate with age in any layer of the Cr; layer a (r^2= 0.009, p=0.71), b (r^2= 0.001, p=0.87), c (r^2= 0.21, p=0.06) or d (r^2= 0.06, p=0.29).

c) Comparisons between raced and non-raced horses

There was no significant difference in the collagen content in the raced and non-raced horses (refer to Figure 3.9).

d) Pattern of the collagen content throughout the depth of the bone

The collagen content throughout layers a to d in both the raced and non-raced horses is shown in Figure 3.9. The variation amongst the layers a to d is significant (p=0.03), being greatest in layer a and equally lowered in layers b-d.

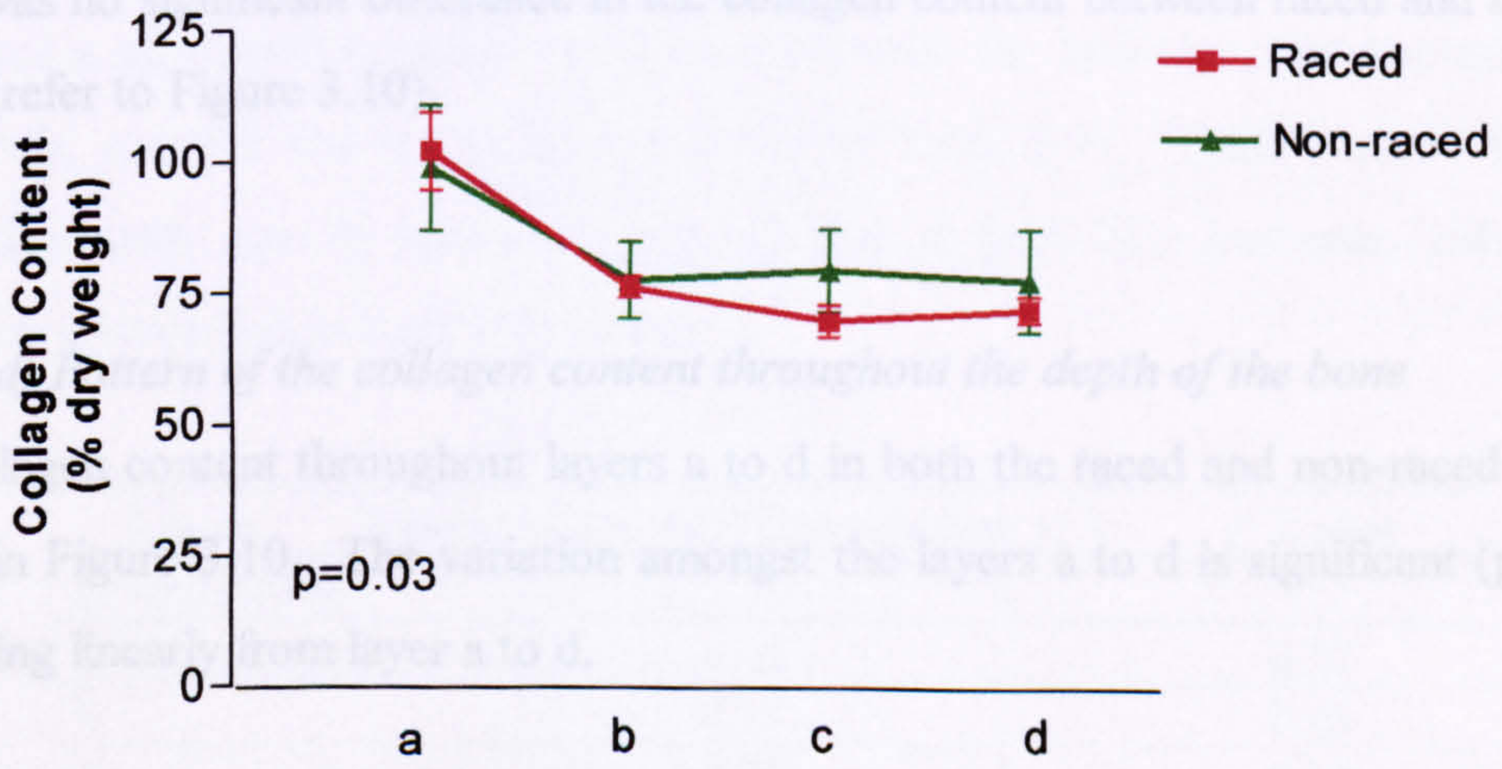


Figure 3.9: The collagen content in Cr of raced and non-raced horses, layers a-d. The variation amongst the layers a to d was significant (p=0.03)

2) C3:

a) Right and left differences

The percentage (%) collagen content was not significantly different between the right and left C3 (layer a-d) of either the raced or non-raced horses (see Appendix Three).

b) Correlation and covariation with age

The collagen content in the horses significantly increased with age in layers b, c and d, although there was no significant correlation in layer a (refer to Table 3.6). There was additionally a significant covariation with age in layer b (p=0.02), layer c (p=0.001) and layer d (p=0.01).

Layer	Linear (Pearson) Correlation (r^2)	P Value
Collagen Content (%)		
a	+0.03	0.46
b	+0.23	**0.04**
c	+0.62	**0.0002**
d	+0.46	**0.001**

Table 3.6: Correlation coefficients of the collagen content and age in the C3 layers a-d.

c) Comparisons between raced and non-raced horses

There was no significant difference in the collagen content between raced and non-raced horses (refer to Figure 3.10).

d) Pattern of the collagen content throughout the depth of the bone

The collagen content throughout layers a to d in both the raced and non-raced horses is shown in Figure 3.10. The variation amongst the layers a to d is significant (p=0.002), decreasing linearly from layer a to d.

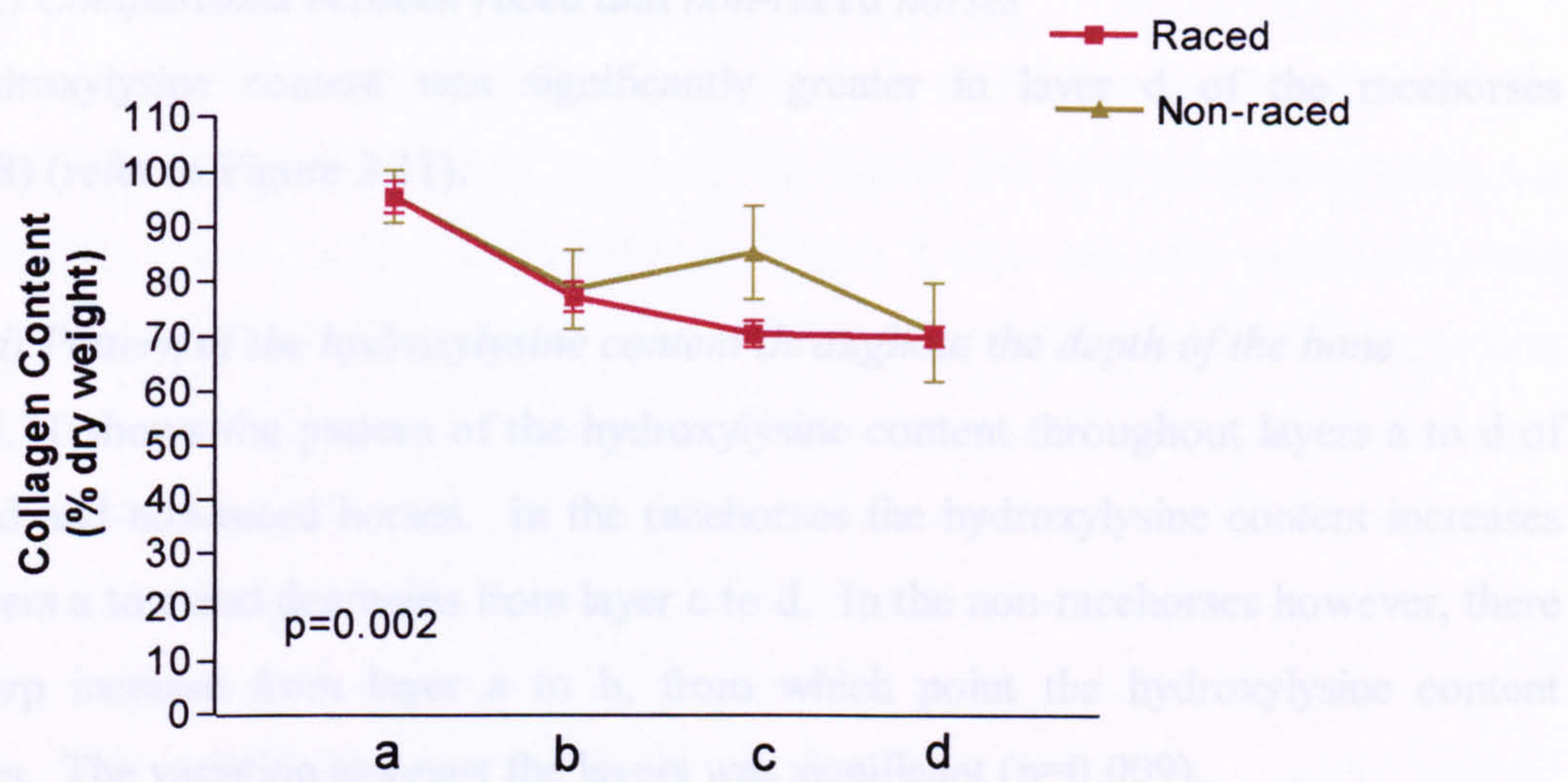


Figure 3.10: The collagen content in C3 of raced and non-raced horses, layers a-d. The variation amongst the layers a to d was significant (p=0.002).

3.1.2.3.5 Hydroxylysine Content

1) Cr:

a) Right and left differences

There were no significant differences between the left and right hydroxylysine content of the Cr in either the raced or non-raced horses (see Appendix Three).

b) Correlation and covariation with age

There was a significant negative correlation between the hydroxylysine content and age in layers b, c and d but not in layer a (refer to Table 3.7). There was no significant covariation with age in layers c (p=0.14) and d (p=0.21), however, there was a significant covariation in layer b (p=0.04).

Layer	Linear (Pearson) Correlation (r^2)	P Value
Hydroxylysine		
a	-0.05	0.32
b	-0.25	**0.03**
c	-0.26	**0.03**
d	-0.30	**0.01**

Table 3.7: Correlation coefficients of the hydroxylysine content and age in the Cr layers a-d.

c) Comparisons between raced and non-raced horses

The hydroxylysine content was significantly greater in layer d of the racehorses (p=0.008) (refer to Figure 3.11).

d) Pattern of the hydroxylysine content throughout the depth of the bone

Figure 3.11 shows the pattern of the hydroxylysine content throughout layers a to d of the raced and non-raced horses. In the racehorses the hydroxylysine content increases from layers a to c and decreases from layer c to d. In the non-racehorses however, there is a sharp increase from layer a to b, from which point the hydroxylysine content decreases. The variation amongst the layers was significant (p=0.009).

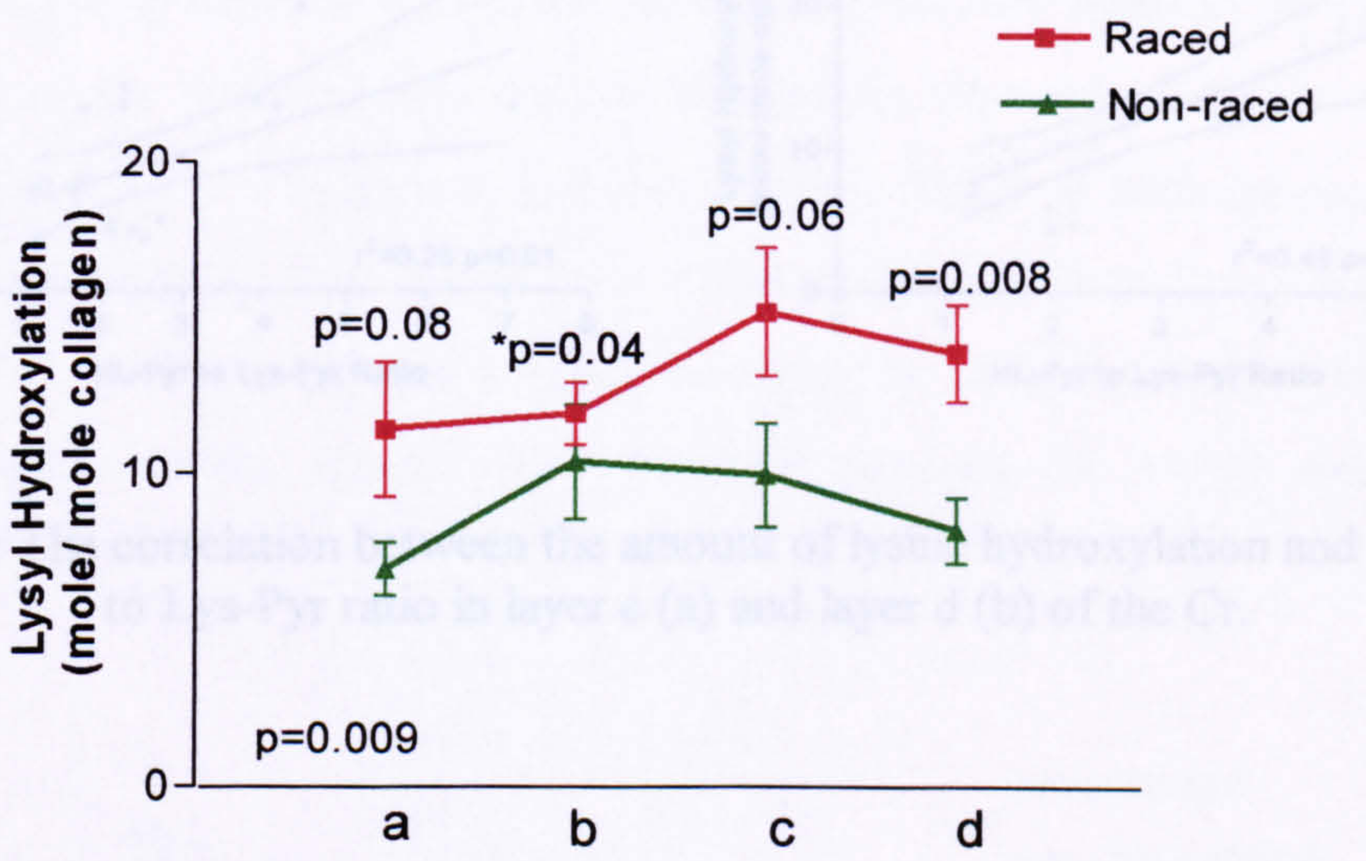


Figure 3.11: The hydroxylysine content throughout the various layers of the raced and non-raced Cr. There was a significant difference between the raced and non-raced horses in layer d (p=0.008). The variation amongst the layers was significant (p=0.009). Note: *p represents the p value given when there was a significant covariation with age.

e) Correlation with the total mature cross-links (HL-Pry plus Lys-Pyr)

There was no significant correlation between the total mature cross-links and hydroxylysine content in layers a, c and d of the Cr in the horses. The hydroxylysine content had a significant covariation with age in layer b (p=0.01), so this layer was not included in the analysis.

f) Correlation with HL-Pyr to Lys-Pyr ratio

A significant correlation was found between the ratio of HL-Pyr and Lys-Pyr and the amount of hydroxylysine in layers c and d; the higher the level of hydroxylation the higher the HL-Pyr to Lys-Pyr ratio (refer to Figure 3.12). No such relationship was found in layer a, however, this may be due to the HL-Pyr to Lys-Pyr ratio being greater and the hydroxylysine content being decreased in the non-raced horses (and vice versa in the racehorses). Due to the lysine hydroxylation content having a significant covariation with age in layer b (p=0.01), this layer was not included in the analysis.

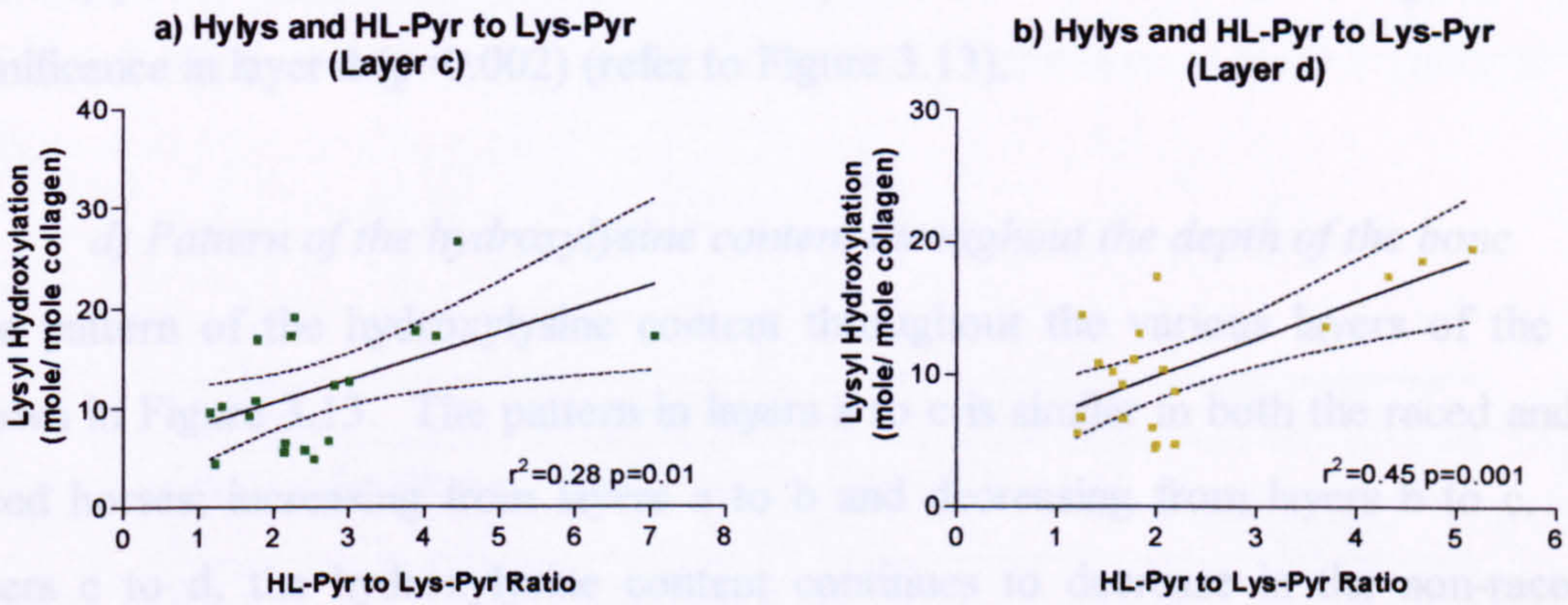


Figure 3.12: The correlation between the amount of lysine hydroxylation and the HL-Pyr to Lys-Pyr ratio in layer c (a) and layer d (b) of the Cr.

2) C3:

a) Right and left differences

There were no significant differences between the left and right hydroxylysine content of the C3 in either the raced or non-raced horses (see Appendix Three).

b) Correlation and covariation with age

There was a significant negative correlation between age and hydroxylysine content in layers c and d but not in layers a and b (refer to Table 3.8). There was no significant covariation with age in these layers.

Layer	Linear (Pearson) Correlation (r^2)	P Value
Hydroxylysine		
a	-0.002	0.84
b	-0.14	0.12
c	-0.23	**0.04**
d	-0.36	**0.01**

Table 3.8: Correlation coefficients of the hydroxylysine content and age in the C3 layers a-d.

c) Comparisons between raced and non-raced horses

As for the hydroxylysine content in the Cr, there was a trend for an increased hydroxylysine content in the raced compared to the non-raced, again reaching significance in layer d (p=0.002) (refer to Figure 3.13).

d) Pattern of the hydroxylysine content throughout the depth of the bone

The pattern of the hydroxylysine content throughout the various layers of the C3 is shown in Figure 3.13. The pattern in layers a to c is similar in both the raced and non-raced horses; increasing from layers a to b and decreasing from layers b to c. From layers c to d, the hydroxylysine content continues to decrease in the non-raced but increases slightly in the raced. The variation amongst the layers was significant (p=0.006).

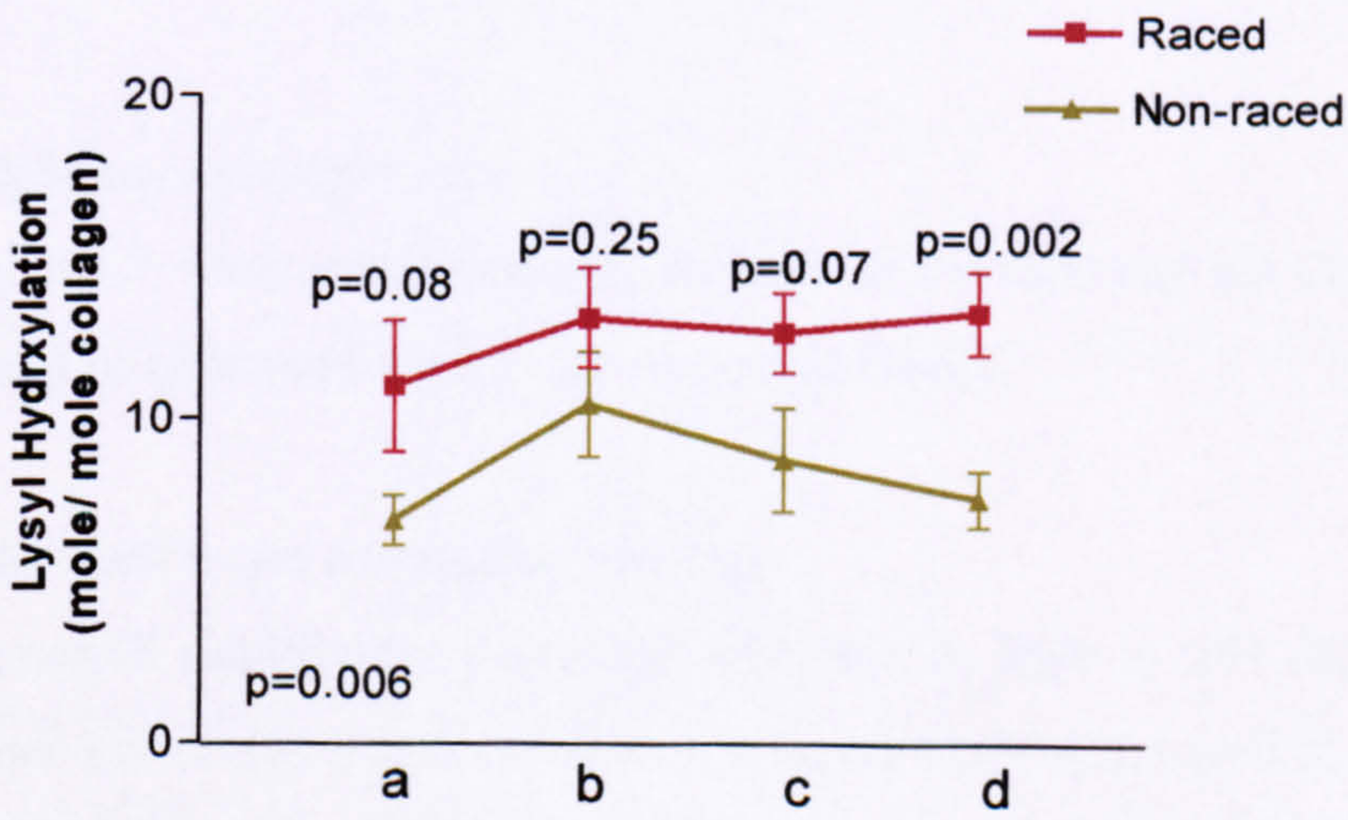


Figure 3.13: The hydroxylysine content throughout the various layers of the raced and non-raced C3. There was a significant difference between the raced and non-raced horses in layer d (p=0.002). The variation amongst the layers was significant (p=0.006). Note: *p represents the p value given when there was a significant covariation with age.

e) Correlation with the total mature cross-links (HL-Pyr plus Lys-Pyr)

There was no correlation between the total mature cross-links and lysine hydroxylation content in layers b, c and d of the C3 in the TB horses. The lysine hydroxylation content had a significant covariation with age in layer a ($p=0.01$), so this layer was not included in the analysis.

f) Correlation with HL-Pyr to Lys-Pyr ratio

There was a significant positive correlation between the ratio of HL-Pyr and Lys-Pyr and the amount of lysine hydroxylation in all layers of the C3. This relationship was significant in layers a, c and d, but did not reach significance in layer b (refer to Table 3.9).

Layer	Linear (Pearson) Correlation (r^2)	P Value
Hydroxylysine and HL-Pyr to Lys-Pyr ratio		
a	+0.33	**0.007**
b	+0.18	0.056
c	+0.33	**0.01**
d	+0.55	**0.0004**

Table 3.9: Correlation coefficients of the HL-Pyr to Lys-Pyr ratio and the lysine hydroxylation content in the C3 layers a-d.

3.1.2.3.6 Collagen Type I Synthesis (PICP)

Quantification of PICP in equine bone extract with the RIA

The standard curve drawn following serial dilution of equine bone extract is shown in Figure 3.14. The bone extract dilution curve is similar to the assay standard curve indicating that the assay is appropriate for the use in quantifying PICP levels in equine bone extract.

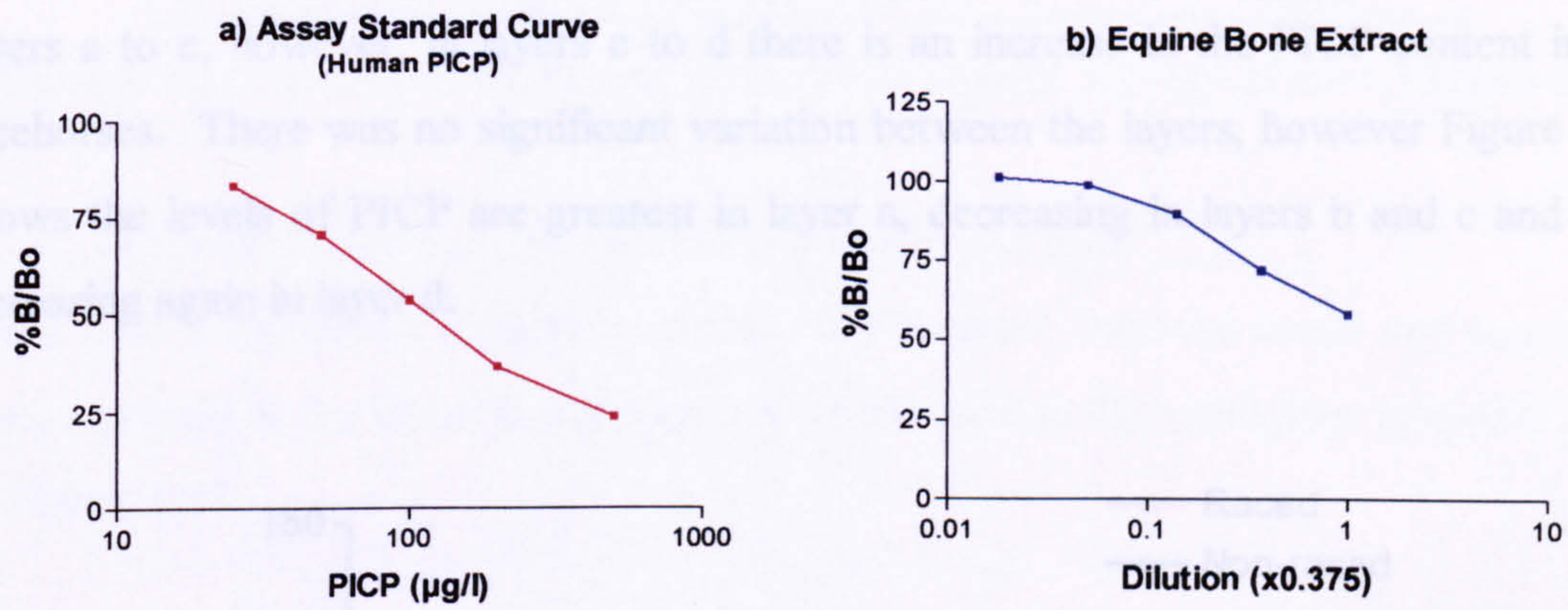


Figure 3.14: Standard curves for human (a) and equine (b) PICP concentration. The logarithmic concentrations of PICP (µg/l) plotted against percentage bound (B) to unbound (Bo) radioactivity.

1) Cr:

a) Right and left differences

The levels of PICP were not significantly different in the right and left Cr (layer a-d) of either the raced or non-raced horses (see Appendix Three).

b) Correlation and covariation with age

The PICP content significantly decreased with age in layer a (r^2= -0.36, p=0.008), however, there was no significant correlation in layers b (r^2= -0.14, p=0.11), c (r^2= -0.13, p=0.12) or d (r^2= -0.11, p=0.16). There was a significant covariation in PICP with age in layer a (p=0.003).

c) Comparisons between raced and non-raced horses

The PICP levels were greater in the raced compared to the non-raced in all layers however, this did not quite reach significance in layers b (p=0.052), c (p=0.057) and d (p=0.06) (refer to Figure 3.15). The p value of layer a (p=0.31) may be explained by the significant covariation with age in this layer.

d) Pattern of the levels of PICP throughout the depth of the bone

Figure 3.15 shows the levels of PICP throughout layers a to d in both the raced and non-raced horses. The pattern of the PICP content is comparable in both groups of horses in layers a to c, however, in layers c to d there is an increase in the PICP content in the racehorses. There was no significant variation between the layers, however Figure 3.15 shows the levels of PICP are greatest in layer a, decreasing in layers b and c and then increasing again in layer d.

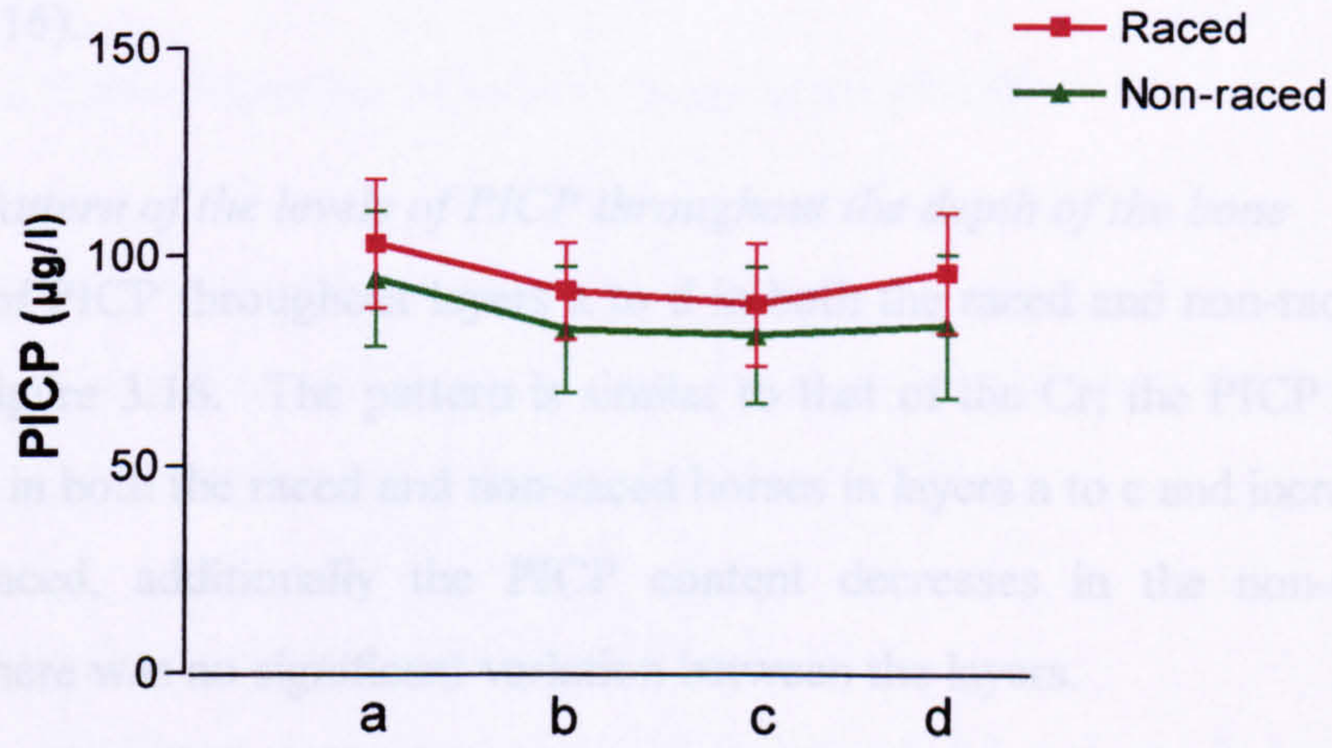


Figure 3.15: The PICP content in the Cr of raced and non-raced horses, layers a-d.

2) C3:

a) Right and left differences

There were no significant differences in the PICP content in the right and left C3 of either the raced or non-raced horses (layer a to d) (see Appendix Three).

b) Correlation and covariation with age

The PICP content significantly decreased with age in all layers of the C3 (refer to Table 3.10). There was a significant covariation with age in layer a (p=0.01), b (p=0.03), and layer c (p=0.03), however, there was no significant covariation in layer d (p=0.09)

Layer	Linear (Pearson) Correlation (r^2)	P Value
PICP		
a	-0.38	**0.005**
b	-0.34	**0.01**
c	-0.33	**0.01**
d	-0.30	**0.01**

Table 3.10: Correlation coefficients of the PICP content and age in the C3 layers a-d.

c) Comparisons between raced and non-raced horses

There was a significant difference in the PICP content between raced and non-raced horses in layer d, the levels being significantly elevated in the racehorses (p=0.02) (refer to Figure 3.16).

d) Pattern of the levels of PICP throughout the depth of the bone

The levels of PICP throughout layers a to d in both the raced and non-raced horses are shown in Figure 3.16. The pattern is similar to that of the Cr; the PICP content being comparable in both the raced and non-raced horses in layers a to c and increasing in layer d in the raced, additionally the PICP content decreases in the non-raced horses. However, there was no significant variation between the layers.

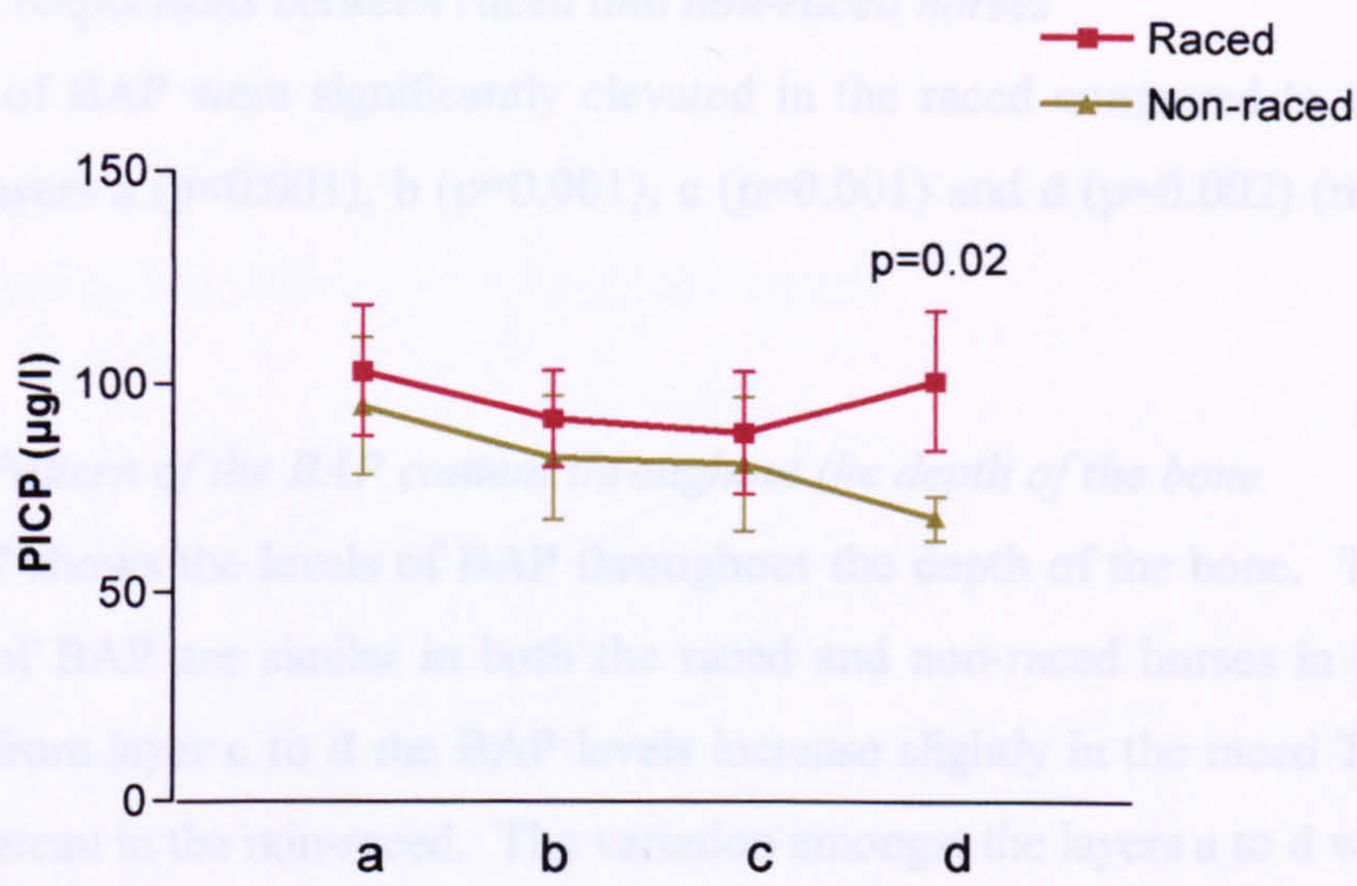


Figure 3.16: The PICP content in the C3 of raced and non-raced horses, layers a-d. There was a significant difference between raced and non-raced in layer d (p=0.02).

3.1.2.3.7 Bone-Specific Alkaline Phosphatase (BAP)

1) Cr:

a) Right and left differences

There were no significant differences in the levels of BAP between the left and right Cr in either the raced or non-raced horses (see Appendix Three).

b) Correlation and covariation with age

There was a significant negative correlation between BAP and age in layers a, b and c but there was no such correlation between BAP and age in layer d (refer to Table 3.11). There was no significant covariation in any of the layers.

Layer	Linear (Pearson) Correlation (r^2)	P Value
BAP		
a	-0.45	**0.002**
b	-0.28	**0.02**
c	-0.22	**0.04**
d	-0.19	0.06

Table 3.11: Correlation coefficients of the BAP activity and age in the Cr layers a-d.

c) Comparisons between raced and non-raced horses

The levels of BAP were significantly elevated in the raced compared to the non-raced horses in layers a ($p<0.001$), b ($p=0.001$), c ($p=0.001$) and d ($p=0.002$) (refer to Figure 3.17).

d) Pattern of the BAP content throughout the depth of the bone

Figure 3.17 shows the levels of BAP throughout the depth of the bone. The pattern in the levels of BAP are similar in both the raced and non-raced horses in layers a to c. However, from layer c to d the BAP levels increase slightly in the raced TB while they begin to plateau in the non-raced. The variation amongst the layers a to d was significant ($p<0.0001$), being greatest in layer a.

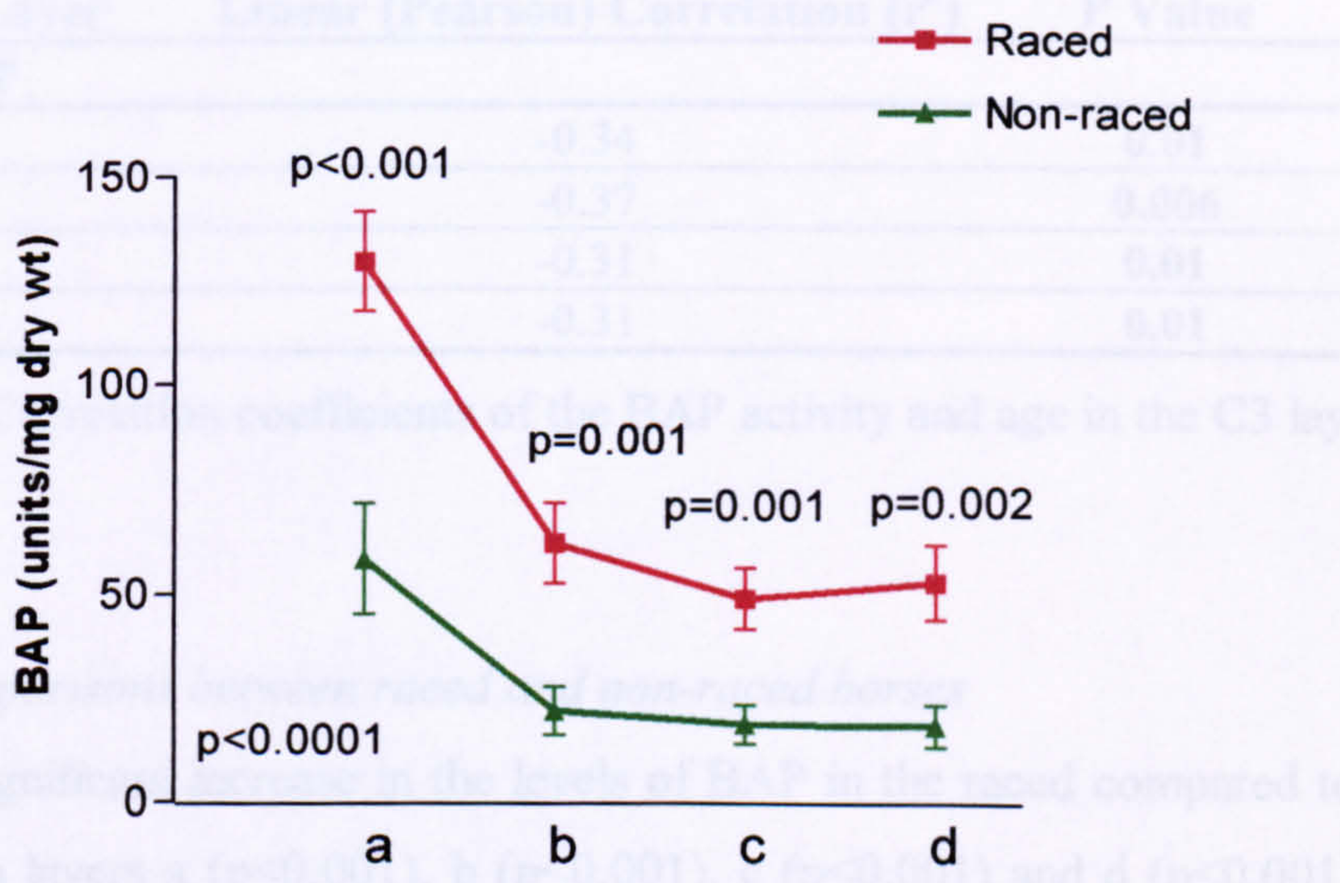


Figure 3.17: The BAP levels in the Cr of raced and non-raced horses, layers a-d. There was a significant difference between the raced and non-raced horses in layers a ($p<0.001$), b ($p=0.001$), c ($p=0.001$) and d ($p=0.002$). The variation amongst the layers a to d was significant ($p<0.0001$).

2) C3:

a) Right and left differences

The levels of BAP in the right and left C3 of raced and non-raced horses (layers a to d) were not significantly different (see Appendix Three).

b) Correlation and covariation with age

There was a significant negative correlation between BAP levels and age in all layers of the C3 (refer to Table 3.12). However, there was no significant covariation with age in any layer.

Layer	Linear (Pearson) Correlation (r^2)	P Value
BAP		
a	-0.34	**0.01**
b	-0.37	**0.006**
c	-0.31	**0.01**
d	-0.31	**0.01**

Table 3.12: Correlation coefficients of the BAP activity and age in the C3 layers a-d.

c) Comparisons between raced and non-raced horses

There was a significant increase in the levels of BAP in the raced compared to the non-raced horses in layers a ($p<0.001$), b ($p<0.001$), c ($p<0.001$) and d ($p<0.001$) (refer to Figure 3.18).

d) Pattern of the BAP content throughout the depth of the bone

The concentrations of BAP throughout the depth of the C3 are shown in Figure 3.18. BAP decrease from layers a to d in both the raced and non-raced horses. The variation amongst the layers a to d is significant ($p<0.0001$), being greatest in layer a.

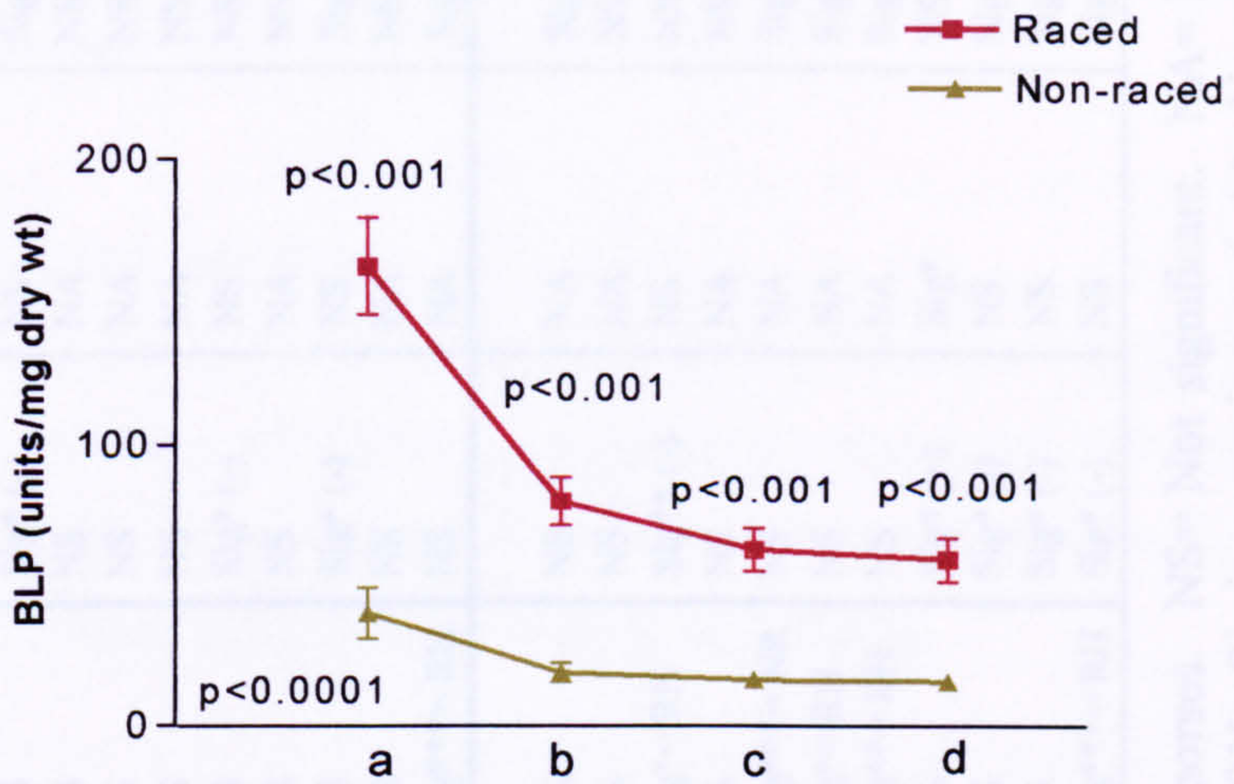


Figure 3.18: The BAP levels in the C3 of raced and non-raced horses, layers a-d. There was a significant difference between the raced and non-raced horses in layers a ($p<0.001$), b ($p<0.001$), c ($p<0.001$) and d ($p<0.001$). The variation amongst the layers a to d was significant ($p<0.0001$).

Marker of Synthesis	Layer a			Layer b			Layer c			Layer d		
	Correlation with Age	Covariation with Age	Significant Difference RH & NR	Correlation with Age	Covariation with Age	Significant Difference RH & NR	Correlation with Age	Covariation with Age	Significant Difference RH & NR	Correlation with Age	Covariation with Age	Significant Difference RH & NR
Cr												
diHLNL	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
HLNL	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
diH:HL	Sig* (-)	Sig**	NS	Sig* (-)	NS	Sig**= RH	NS	NA	NS	Sig* (-)	NS	Sig*= RH
HL-Pyr	NS	NA	Sig*= NR	NS	NA	NS	NS	NA	NS	NS	NA	NS
Lys-Pyr	NS	NA	NS	Sig* (+)	NS	Sig***= NR	Sig** (+)	Sig*	NS	NS	NA	NS
HP:LP	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
Im:Mat	NS	NA	NS	NS	NA	NS	NS	NA	NS	Sig* (-)	NS	NS
Col Con	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
Hyl-Lys	NS	NA	NS	Sig* (-)	Sig*	NS	Sig* (-)	NS	NS	Sig* (-)	NS	Sig**= RH
PICP	Sig** (-)	Sig**	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
BAP	Sig** (-)	NS	Sig***= RH	Sig* (-)	NS	Sig***= RH	Sig* (-)	NS	Sig***= RH	NS	NA	Sig***= RH
C3												
diHLNL	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	Sig*= RH
HLNL	Sig** (+)	Sig*	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
diH:HL	Sig** (-)	NS	Sig**= RH	NS	NA	Sig*= RH	Sig* (-)	NS	Sig*= RH	Sig** (-)	NS	NS
HL-Pyr	Sig* (+)	NS	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
Lys-Pyr	Sig*** (+)	Sig**	NS	Sig** (+)	NS	Sig***= NR	Sig* (+)	NS	Sig***= NR	NS	NA	Sig***= NR
HP:LP	NS	NA	NS	NS	NA	NS	NS	NA	Sig*= RH	NS	NA	Sig*= RH
Im:Mat	NS	NA	NS	Sig* (-)	NS	NS	NS	NA	Sig**= RH	NS	NA	Sig***= RH
Col Con	NS	NA	NS	Sig* (+)	Sig*	NS	Sig** (+)	Sig**	NS	Sig* (+)	Sig*	NS
Hyl-Lys	NS	NA	NS	NS	NA	NS	Sig* (-)	NS	NS	Sig* (-)	NS	Sig**= RH
PICP	Sig** (-)	Sig*	NS	Sig* (-)	Sig*	NS	Sig* (-)	Sig*	NS	Sig* (-)	NS	Sig*= RH
BAP	Sig* (-)	NS	Sig***= RH	Sig** (-)	NS	Sig***= RH	Sig* (-)	NS	Sig***= RH	Sig* (-)	NS	Sig***= RH

Table 3.13: Summary of the markers of bone synthesis in the Cr and C3 layers a-d of raced and non-raced horses. NS= Not significant. NA= Not applicable. Sig= Significant results= *(P<0.05), **(P<0.01), ***(P<0.001). NR= Sig increase in non-raced. RH= Sig increase in raced. (-)= Negative correlation with age. (+)= Positive correlation with age. Im:Mat= Immature to mature cross-link ratio. diH:HL= diHLNL to HLNL ratio. HP:LP= HL-Pyr to Lys-Pyr ratio. Col Con =% Collagen content.

3.1.2.4 Discussion

Relationships with Age

The ages of the horses in this study varied from 3 to 22 years, with the widest age range being evident in the non-raced horses. A correlation was found between age and several of the markers of bone collagen synthesis, with these relationships being evident throughout the cortical and trabecular regions of the bone.

The correlation between the immature, and mature, cross-link content and age varied between layers of the Cr and C3, but generally, age correlated positively with the mature, and to a lesser extent, the immature cross-links. The increase in mature collagen cross-links with age is a normal ageing process and has been demonstrated in ageing human collagen (Bailey, Paul et al. 1998; Wang, Shen et al. 2002). However, the positive correlation between the immature cross-links and age is less easy to explain since it would be expected that as the immature cross-links form mature cross-links with age their numbers would decline. One explanation for this is that in this study the data for raced and non-raced ages were pooled and, as such, this correlation may be reflecting the presence of newly formed collagen during the constant remodelling process of bone in the racehorses, rather than truly reflecting an age effect.

The PICP and BAP content were found to negatively correlate with age in most layers of the Cr and C3. These data are consistent with those of Price *et al.*, (1995) who demonstrated a fall in serum PICP and serum ALP and BAP in horses with age (Price, Jackson et al. 1995). Additionally, Blumsohn *et al.*, (1994) have also shown in humans that levels of BAP and PICP were highest during the rapid bone modelling associated with the pubertal growth spurt in girls and deceased significantly thereafter (Blumsohn, Hannon et al. 1994).

Regardless of the effect of age on collagen synthesis, high-intensity exercise as experienced by racing TBs, appeared to have a significant effect on the collagen synthesis in the subchondral and trabecular regions of the radial and third carpal bones.

The Effects of Exercise

In both the Cr and C3, the cross-link composition is different between the two groups of horses. The HL-Pyr and Lys-Pyr content was significantly less in the racehorses compared to non-racehorses and, although there were no significant differences in the diHLNL and HLNL content, the ratio of immature to mature cross-links was greater in the racehorses compared to the non-racehorses, suggesting that high-intensity exercise may be inducing elevated collagen turnover. These findings are supported by a study on the cartilage of strenuously exercised two year-old TBs by Brama *et al.*, (2000), in which the exercise group had significantly less HL-Pyr cross-link content compared to non-exercised controls (Brama, Tekoppele et al. 2000). Since mature trivalent cross-links are known to enhance the strength of the tissue, a reduction or disruption in these cross-links may result in a less resilient bone.

The increased HL-Pyr to Lys-Pyr ratio apparent in the racehorses is a direct consequence of the increased lysyl hydroxylation in these animals, this being corroborated by the positive correlation between the lysyl hydroxylation and HL-Pyr to Lys-Pyr ratio in this study. This finding is further supported by the increase in diHLNL to HLNL ratio, the precursors of HL-Pyr and Lys-Pyr.

A high level of lysyl hydroxylation is characteristic of immature tissues, with chick embryonic bone being reported to contain twice as much hydroxylysine as adult bone (Miller, Martin et al. 1967; Strawich and Glimcher 1983). This increase in hydroxylysine, and corresponding increase in cross-link ratios, therefore indicates that racehorse bone consists of a more immature matrix, consistent with a higher turnover of the collagenous matrix. In addition, increased levels of lysyl hydroxylation have been reported during fracture repair of avian bone (Glimcher, Shapiro et al. 1980). This post-translational modification of the collagen fibrils in the raced bone could therefore be an attempt at a repair mechanism, to combat the possible accumulation of microfractures resulting from the increased mechanical load during high-intensity exercise.

Increased lysine hydroxylation has also been documented in certain forms of osteogenesis imperfecta (brittle bones) (OI) (Kirsch, Krieg et al. 1981) and OP (Knott, Whitehead et al. 1995; Kowitz, Knippel et al. 1997) and is believed to be responsible for

the formation of thin collagen fibrils as a consequence of retarded triple helix formation (Torre-Blanco, Adachi et al. 1992). Thus, the increasing hydroxylation of the lysine residues in the raced bone collagen could similarly lead to thinner fibrils, and hence reduce the strength of the bone. Additionally, increased hydroxylation is known to result in fewer pyrrole cross-links. Pyrrole cross-links have been shown to contribute more to the tensile strength of bone than the pyridinoline cross-links (Knott, Whitehead et al. 1995), which is speculated to be due to the pyrrole forming an interfibrillar cross-link, in contrast to pyridinoline forming an intrafibrillar cross-link and consequently having a greater effect on the mechanical properties of the fibre (Knott and Bailey 1998). However, in this study because of a lack of tissue, the pyrrole content was not quantified in the bone of the TBs and hence it is difficult to investigate this issue further. Although, if an increased lysyl hydroxylation does result in less pyrrole (the cross-link suggestive to contribute most to the mechanical strength of bone) this may further support the notion that the bone in the Cr and C3 of racehorses is weaker.

No relationship was found to exist between the total mature cross-links (HP-Pyr plus Lys-Pyr) and the amount of lysyl hydroxylation in the racehorses, despite the increase in the level of lysine hydroxylation. HP-Pyr and Lys-Pyr can only be formed if the lysine in the telopeptides is hydroxylated. The lack of concomitant increase of the total mature cross-links with increasing lysyl hydroxylation of the triple helix indicates independent control of telopeptide and triple helical lysine hydroxylation. These data support the recent suggestion that two classes of lysyl hydroxylases exist, one for the triple helix and one for the telopeptide (Bank, Robins et al. 1999; Brama, Bank et al. 2001).

The increase in cross-links and lysyl hydroxylation was not solely isolated to the cortical layer, but differences between the two groups of horses were also observed in the deeper, trabecular regions of the bone, particularly layers c and d. Mansell *et al.*, (1998) have shown that in the bone of human OA femoral heads, both subchondral and trabecular bone collagen metabolism is increased (Mansell and Bailey 1998), demonstrating that not only may subchondral bone be important in the progression of joint diseases such as OA but that trabecular bone may also, which is further supported by the data in this study. The levels of cross-links, particularly the immature cross-links, were found to be greater in the trabecular layers compared to the cortical layer

presumably reflecting the higher turnover rate and hence the more immature state of the trabecular bone (Eyre, Dickson et al. 1988).

The increased PICP content (although not significant) and significantly increased BAP content in the racehorses further demonstrate that high-intensity exercise induces elevated bone formation. Studies have previously shown that the concentration of PICP and BAP are increased in treadmill exercised horses compared to controls (Price, Jackson et al. 1995; Hiney, Potter et al. 2000; Kawcak, McIlwraith et al. 2000). However, in those studies BAP and PICP were quantified in serum and since type I collagen is present in many tissues these values may not solely reflect bone activity. Additionally, these values were obtained on treadmill-exercised horses, which is not equivalent to the galloping on grass or dirt racetracks undertaken by the racehorses in this study. Therefore, this study shows for the first time, that the concentrations of PICP and BAP are increased with conventional racing and race-training.

Similar to the cross-link and hydroxylysine content, increases in the PICP and BAP content were not isolated to the cortical layer, but elevated levels were also present in the trabecular regions of the bone, particularly within layers c and d. Although in contrast to the cross-link and hydroxylysine content, these concentrations were greatest in the cortical regions, declining slightly throughout the trabecular regions of the bone. Interestingly however, between layers c and d the PICP and ALP concentrations increased quite sharply in the racehorses, especially within the C3, thus reflecting further the high rate of remodelling in the trabecular regions of the racehorse bone.

The collagen content in the Cr and C3, although not significantly different in the two groups, was higher in the non-raced compared to the racehorses. This may be attributable to the relationship found to exist between the collagen content and age, since the greatest variation with age is within the non-racehorses, but this relationship was only found to exist in layer c of the Cr. It is possible therefore, that the loss of collagen content in the racehorses is a reflection of the damage and breakdown to the collagenous matrix due to the increased mechanical load. Salem *et al.*, (1993) reported a lower hydroxyproline concentration in the femoral necks of eight-week-old rats after a moderate exercise regime of 10 weeks duration (Salem, Zernicke et al. 1993).

Additionally a reduction in the collagen content has been documented in the cartilage of treadmill exercised compared to non-exercised horses (Murray, Birch et al. 2001). Hence while bone collagen is being synthesised, which is reflected in the PICP results, the increased mechanical load may be causing a breakdown of this collagen.

3.1.3 Bone Collagen Degradation

3.1.3.1 Introduction

3.1.3.1.1 Matrix Metalloproteinases 2 and 9 (MMP-2 &-9)

The importance of MMPs in the normal physiological turnover of collagen is well known. However, despite the normal homeostatic role of MMPs in connective tissue metabolism, these enzymes have been shown in human and experimental animals to lead to the destruction of the ECM in diseased tissues and hence their quantification can provide information on the level of collagen degradation in the tissue (Mansell, Tarlton et al. 1997; Tarlton, Whiting et al. 2000; Riley, Curry et al. 2002). MMPs have also been considered for some time to be involved in the pathogenesis of equine joint disease. Elevated levels of MMP-2 and-9 (gelatinase A and gelatinase B respectively) have been documented in equine OA and OC synovial fluid and cartilage (Brama, TeKoppele et al. 1998; Clegg and Carter 1999; Al-Hizab, Clegg et al. 2002), but to date, their levels in equine bone have not been quantified.

The primary role of MMP-2 and -9 is to degrade denatured collagen and type IV collagen. However, MMP-2 has been found to cleave the same Gly_{775}-Leu/Ile_{776} site in the collagen α chains as collagenase-1 (MMP-1), producing the characteristic ¾ (TC^A) and ¼ (TC^B) fragments typical of collagenolytic activity (Aimes and Quigley 1995; Patterson, Atkinson et al. 2001). It is not known to what extent MMP-2 has collagenolytic activity *in vivo*. However, Kerkvliet *et al.*, (1999) found a positive correlation between MMP-2 activity (but not with collagenase activity) and degradation of soft tissue explants (Kerkvliet, Docherty et al. 1999). Thus suggesting that not only is MMP-2 a marker of triple helical collagen degradation but it may also be an effector.

MMP-9 is primarily an inflammatory cell enzyme, being secreted by monocytes, macrophages and neutrophils during inflammation, but its involvement in bone development has been documented (Ahrens, Koch et al. 1996; Tarlton, Vickery et al. 1997). In a study by Vu *et al.*, (1998), mice in which the MMP-9 gene had been inactivated, displayed a specific defect in endochondral bone formation, characterised by an accumulation of hypertrophic cartilage at the skeletal growth plates. Although it is

not known fully how MMP-9 activity regulates endochondral ossification, its importance in bone formation during maturation is apparent.

As with many of the markers of collagen synthesis, MMP activity has been shown to decline with increasing age. In a study by Brama *et al.*, (1998) the MMP activity in normal equine adult (mean age 11.3 years) MCP joint synovial fluid was found to be almost half of that in normal juvenile (5 and 11 months) MCP joint synovial fluid, with the youngest foals showing the highest MMP activity (Brama, TeKoppele et al. 1998).

3.1.3.1.2 Tissue Inhibitors of Matrix Metalloproteinases (TIMPs)

In order to prevent excessive MMP activity, and the resulting degradation of collagen in bone, MMPs are controlled by several natural inhibitors including TIMPs. The dynamic equilibrium between TIMPs and active MMPs is therefore an important regulatory mechanism of MMP activity. In addition to maintaining control over the activity of MMPs, specifically TIMP-1 and TIMP-2 are able to bind directly to the hemopexin domain of MMP-9 and MMP-2 respectively, exerting further control over the activation process. A deficiency of TIMP-1 and-2 relative to levels of MMP-9 and –2 has been demonstrated in OA human cartilage (Dean, Azzo et al. 1987) and bone (St'ovickova, Hulejova et al. 2002) and their presence in normal and septic equine synovial fluid has been demonstrated (Clegg, Coughlan et al. 1998). Quantification of TIMP-1 and –2 activity is therefore essential in order to determine net MMP-9 and –2 activity in tissue.

3.1.3.1.3 Collagenase Cleaved Type I and II Collagen

As already mentioned, the collagenases are capable of cleaving the fibrillar collagens at a single site (Gly_{775}-Leu/Ile_{776}) within each α chain of the triple helical collagen molecule, approximately three quarters of the distance from the amino-terminal end of each chain, resulting in the generation of ¾ and ¼ length collagen fragments (Miller, Harris et al. 1976). Recently, a rabbit polyclonal antibody, namely the COL2-3/4C_{short} antibody, has been developed which recognises the neoepitope that is created at the C terminus (¾

fragment) of the primary cleavage site (COL2-3/4C_{short}) by collagenase (Billinghurst, Dahlberg et al. 1997), thus allowing the specific quantification of the amount of COL2-3/4C_{short} within the tissue and hence reflecting the amount of degraded collagen.

A commercially available two-step competitive immunoassay (Ibex Diagnostics, USA) has been developed to quantify the amount of COL2-3/4C_{short} generated by the cleavage of types I and II collagens by collagenases. The assay is primarily designed for the quantification of human collagenase cleaved type II collagen (Billinghurst, Dahlberg et al. 1997; Stoop, van der Kraan et al. 1999; Billinghurst, Wu et al. 2000; Dahlberg, Billinghurst et al. 2000) but shows cross-reactivity with other species including the horse and levels in equine cartilage (Billinghurst, Buxton et al. 2001; Laverty, Okouneff et al. 2002) and sera (Billinghurst, Brama et al. 2003) have been documented. The antibody also shows affinity for cleaved human type I collagen (Sukhova, Schonbeck et al. 1999), but to date the cross-reactivity of the antibody to type I bone collagen and, specifically equine type I bone collagen has not been demonstrated. Hence part of this study was to investigate the use of this assay in the quantification of cleaved type I collagen in equine bone extract.

The influence of age on the levels of COL2-3/4C_{short} in normal human ankle cartilage has been quantified in 30 adults (age 16-75 years) (Aurich, Poole et al. 2002). The results showed that there was no correlation with the levels of COL2-3/4C_{short} and age. However, this study does not reflect the influence, if any, on immature individuals.

3.1.3.2 Materials and Methods

Details of the materials and solutions used in the following methods are listed in Appendices Four and Five respectively.

3.1.3.2.1 Equine Samples

The following analyses on markers of bone collagen degradation were obtained on the same samples analysed for markers of bone collagen synthesis (refer to section 3.1.2.2.1), unless otherwise stated.

3.1.3.2.2 Gelatin Zymography (for Gelatinase A (MMP-2) and Gelatinase B (MMP-9) quantification)

Aliquots of the soluble protein extract were thawed to room temperature and reconstituted in non-reducing sample buffer at a ratio of 2:1. Biorad Mini Protean II casting apparatus was set up with 1mm spacers. 10% gelatin resolving gel solution was poured into the casting apparatus. Gels were overlaid with dH_20 and left to polymerise for 45 minutes at room temperature. The dH_20 was then removed and stacking gel was poured on top of the set resolving gel. 15 well sample combs were then inserted, ensuring no air bubbles were present and the gel was left to polymerise at room temperature for a further 45 minutes. The samples, standards (MMP-2, Biogenesis, UK) and rainbow protein molecular weight markers (Mr=14-200kDa, Amersham, UK) were loaded into the sample wells and the gels electrophoresed at 100V and 20mA/ gel. Following electrophoresis, the gels were washed in 2.5% (v/v) Triton X-100 (BDH) for 30 minutes to displace the SDS and then incubated for 48hrs at 37°C in MMP proteolysis buffer. After staining with Coomassie Brilliant Blue R250 (Aldrich Chemical Company, UK) (0.25%) then destained for 1 hour, zones of proteolytic activity were observed.

3.1.3.2.3 Reverse Gelatin Zymography (for MMP inhibitors, TIMP-1 and –2, quantification)

Equine Samples

Table 3.14 shows the horses used to quantify the TIMP activity.

Raced	Age	Non-raced	Age
R3	8	**5**	9
R4	4	**6**	12
R5	6	**7**	10
R6	7	**9**	8
R7	7	**10**	NA
R8	7	**11**	3

Table 3.14: The horses used to quantify the TIMP activity and their ages (NA= Not Known).

Reverse Zymography

This method is one based on that detailed by (Oliver, Leferson et al. 1997). Gelatin and recombinant MMP-2 (Calbiochem, UK) was incorporated into a 12.5% resolving gel, to produce a final concentration of 1.3mg/ml and 70µg/l respectively. The recombinant MMP-2 resolving gel solution (see Appendix Five) was immediately poured into the apparatus as described above. The TIMP-2 standard (human rheumatoid synovial fibroblast, Calbiochem, UK) was mixed 1:2 with non-reducing sample buffer and the bone extract (refer to Chapter 2, section 2.2.3.1) was mixed 2:1 with sample buffer and both were loaded onto the MMP-2 resolving gels. The gels were then electrophoresed as described above. The gels were washed in 2.5% (v/v) Triton X-100 (BDH) for 90 minutes. Gels were then washed thoroughly with dH_2O and incubated at 37°C in 20ml MMP proteolysis buffer and 4-(2-Aminoethyl)- benzenesulfonyl fluride (AEBSF) at a final concentration of 50µM for approximately 48hrs. AEBSF was added to irreversibly block serine proteases; activation of these serine proteases in TIMP gels performed early in this study were found to be affecting the intensity of the bands and hence elimination was necessary in order to fully quantify the expression of TIMP in the samples. The gels were subsequently stained and de-stained as described previously. The areas of inhibition (mainly TIMP-2) were marked as dark staining areas on a clear background.

10% SDS-PAGE Gel

Identical samples tested by reverse zymography were also analysed by non-reducing SDS-PAGE (see Appendix Five) to confirm that the bands seen on the reverse zymogram were due to TIMP activity and not the presence of other proteins of similar molecular weight.

3.1.3.2.4 MMP-2 and-9 and TIMP-2 Quantification

Quantification of MMP and TIMP activity was achieved by optical scanning of the gels (Agfa Studioscan II Colour Scanner and Photoshop imaging software) and subsequent computer analysis of the band intensities (NIH Image 1.5) as described previously (Tarlton and Knight 1996). The enzyme levels were expressed as a percentage of activity of the MMP-2 and TIMP-2 standard, such that comparisons could be made between gels.

3.1.3.2.5 Collagen II 3/4C$_{short}$ Assay for Quantification of Collagenase Cleaved Type I and Type II Collagen

Equine Samples

The differences between the right and left carpi in the raced and non-raced horses in the other markers of synthesis and degradation quantified were minimal (see Appendix Three). Therefore the amount of COL2-3/4C$_{short}$ epitope was only obtained in the left Cr and C3 of each cadaver (raced n=9, non-raced n=11).

Tissue Preparation

A proportion of the pulverised bone was decalcified in 0.5m EDTA (tetrasodium salt) pH 7.5 for 2 days at 4°C (at a concentration of 10mg/5ml EDTA) with a change of EDTA after the 1st day, and subsequently washed in dH_2O (x2) to remove the EDTA. The demineralised bone was incubated overnight at 37°C with 1mg/50-75mg bone (w/w) of α-chymotrypsin in 50mM Tris-HCL, pH 7.6 to solubilise the cleavage epitope. The α-chymotrypsin activity was inhibited with 20µl (160µg/ml final concentration) of N-tosyl-l-phenylalanine-chloromethyl ketone (TPCK). After 20 minutes in TPCK at 37°C, the

samples were centrifuged, the supernatant removed and subsequently frozen at -20°C until required.

To enable the expression of the amount of COL2-3/4C_{short} neoepitope as a percentage of the total collagen content (representing the % cleaved collagen), the remaining bone residues were weighed and hydrolysed in 6N HCL (at a concentration of 5mg/ ml HCL), at 110°C overnight and freeze dried in an acid freeze drier overnight. The samples were then rehydrated in 1ml dH_2O, and the hydroxyproline content quantified as described previously (refer to section 3.1.2.3).

Collagen II 3/4C_{short} ELISA

The collagen II 3/4C_{short} assay is a two-step competitive immunoassay in a 96-well plate format utilising two short peptide epitopes (peptide 287 and 378) that are recognised by the COL2-3/4C_{short} rabbit antibody (Billinghurst, Dahlberg et al. 1997). KLH-287 peptide conjugate is coated on the plate. The COL2-3/4C_{short} rabbit antibody is added and binds to either the KLH-287 conjugate on the plate or to free 378 peptide (standards) or to the endogenous neoepitope (samples). After washing, goat anti-rabbit horseradish peroxidase (HRP) conjugate is then added which binds to any COL2-3/4C_{short} rabbit antibody on the plate. Tetra-methylbenzidine (TMB) substrate buffer is added and then HRP degrades H_2O_2 and oxidises TMB to form a blue product. The reaction is stopped and signal amplified with an acid, which converts the product from a blue to a yellow colour that can be quantified at 450nm. The optical density (OD) at 450nm is inversely proportional to the amount of COL2-3/4C_{short} epitope.

3.1.3.2.6 Statistical Analysis

Statistical analysis was performed using SPSS v11.5 (SPSS Inc. USA) and Graphpad Prism v3.0 (Graphpad Software Inc. USA), as detailed in Chapter Two section 2.2.1.5.

3.1.3.3 Results

3.1.3.3.1 Gender

a) Total MMP-2 levels (Cr layers a and d)

There was no significant difference in the total MMP-2 levels between females (10.98+/-3.7) and males (17.41+/-4.1) in layer a ($p=0.43$) and between females (11.12+/-8.1) and males (11.19+/-3.1) in layer d ($p=0.62$).

b) Percentage cleaved collagen (C3 layers b and c)

Additionally, there was no significant difference in the percentage cleaved collagen between females (1.58+/-0.62) and males (1.81+/-0.30) in layer b ($p=0.94$) and between females (2.09+/-0.58) and males (1.55+/-0.22) in layer c ($p=0.31$).

3.1.3.3.2 MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) Levels in Equine Bone

Gelatin zymography clearly showed the presence of MMP-2 and –9, both pro and active, in the Cr and C3 of raced and non-raced horses and throughout the depth of the bone (layers a-d) (see Figure 3.19).

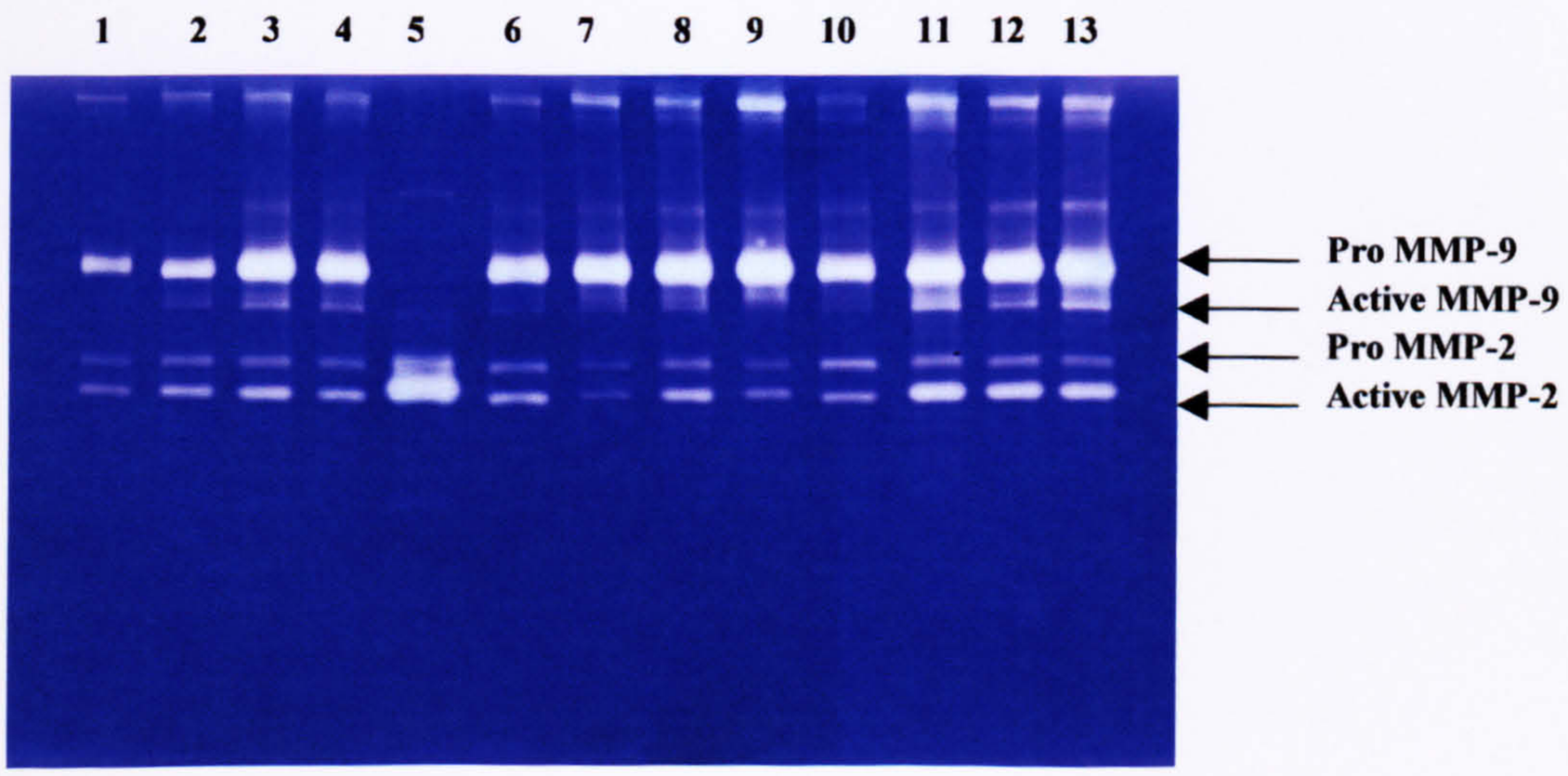


Figure 3.19: An example of a gelatin zymogram of equine bone extract. Lanes 1 and 2 contain extract from the Cr of non-raced layer d. Lanes 3, 4 and 6 contain extract from the Cr of raced layer d. Lanes 7-11 contain extract from the C3 of non-raced layer a. Lanes 12 and 13 contain extract from the C3 of raced layer a. Lane 5 contains the MMP-2 standard containing proactive and active forms of MMP-2.

3.1.3.3.3 Total MMP-2 Levels

The following results are expressed as the total MMP-2 levels. The total MMP-2 levels relates to the amount of MMP-2 expression as it accounts for all forms MMP-2, whether in the pro or active form.

1) Cr:

a) Right and left differences

The levels of total MMP-2 were not significantly different in the right and left Cr (layers a to d) of either the raced or non-raced horses (see Appendix Three).

b) Correlation and covariation with age

Total MMP-2 had a significant negative correlation with age in the horses in layer a, layer b and layer c of the Cr (refer to Figure 3.20). However, no correlation with age was apparent in layer d (r^2= -0.17, p=0.08). There was a significant covariation with age in layers a (p=0.004) and layer c (p=0.04), this was not apparent in layer b (p=0.13).

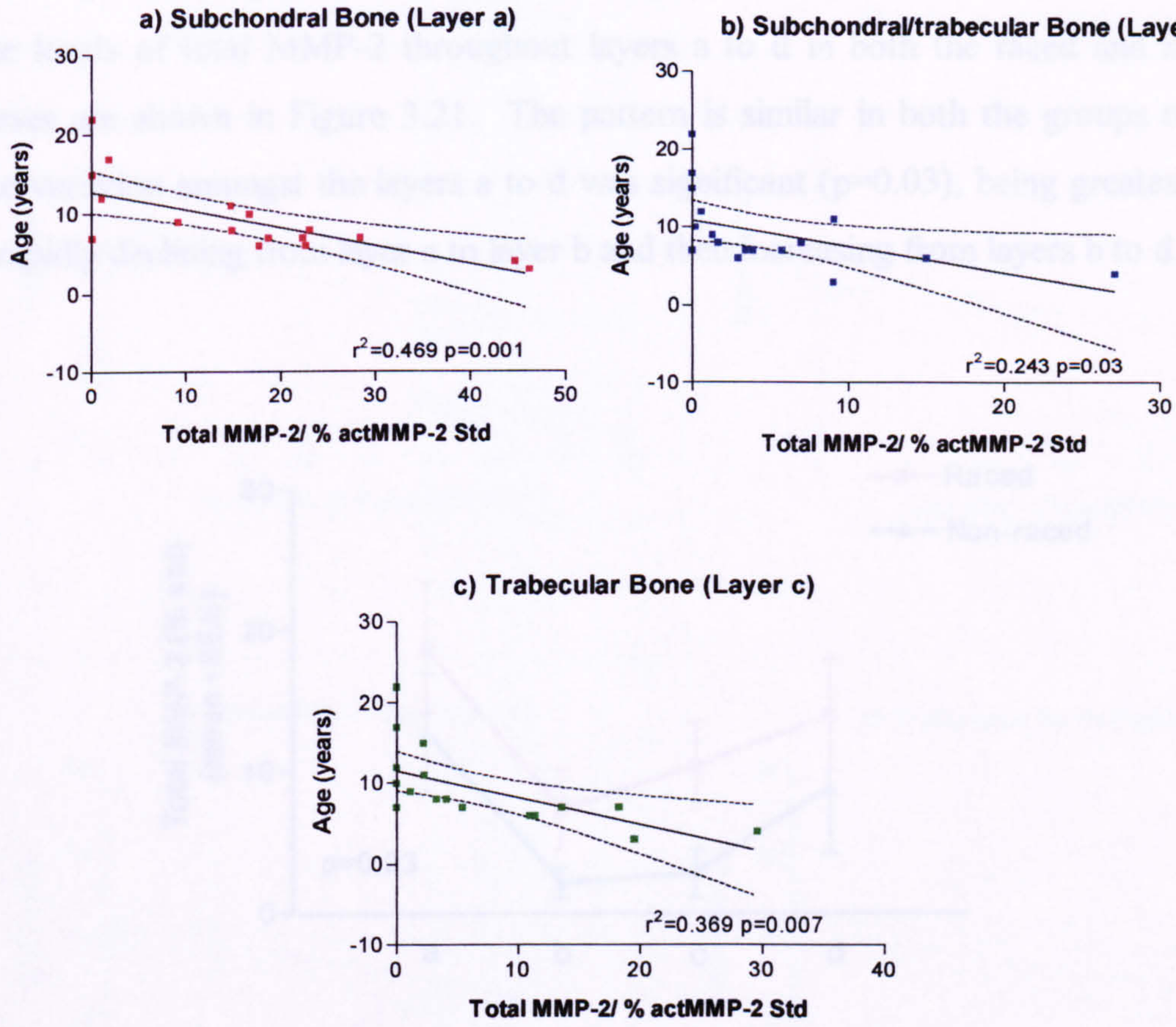


Figure 3.20: The correlation between age and the quantity of activated MMP-2 in the Cr; a) layer a, b) layer b and c) layer c.

c) Comparisons between raced and non-raced horses

There was no significant difference in the levels of total MMP-2 between the raced and non-raced horses in all layers (a-d) of the Cr (refer to Table 3.15)

Layer	Raced	Non-raced	P Value
a	18.78 +/-4.58	12.72 +/-4.3	*P=0.004
b	7.22 +/-2.89	2.14 +/-1.04	P=0.11
c	10.34 +/-3.16	2.75 +/-1.71	*P=0.04
d	13.99 +/-3.92	8.86 +/-4.75	P=0.42

Table 3.15: Total MMP-2 activity in the Cr layers a-d of raced and non-raced horses (mean %std +/- SEM). Note: *P represents the p value given when there was a significant covariation with age.

d) Pattern of the MMP-2 levels throughout the depth of the bone

The levels of total MMP-2 throughout layers a to d in both the raced and non-raced horses are shown in Figure 3.21. The pattern is similar in both the groups of horses. The variation amongst the layers a to d was significant (p=0.03), being greatest in layer a, rapidly declining from layer a to layer b and then increasing from layers b to d.

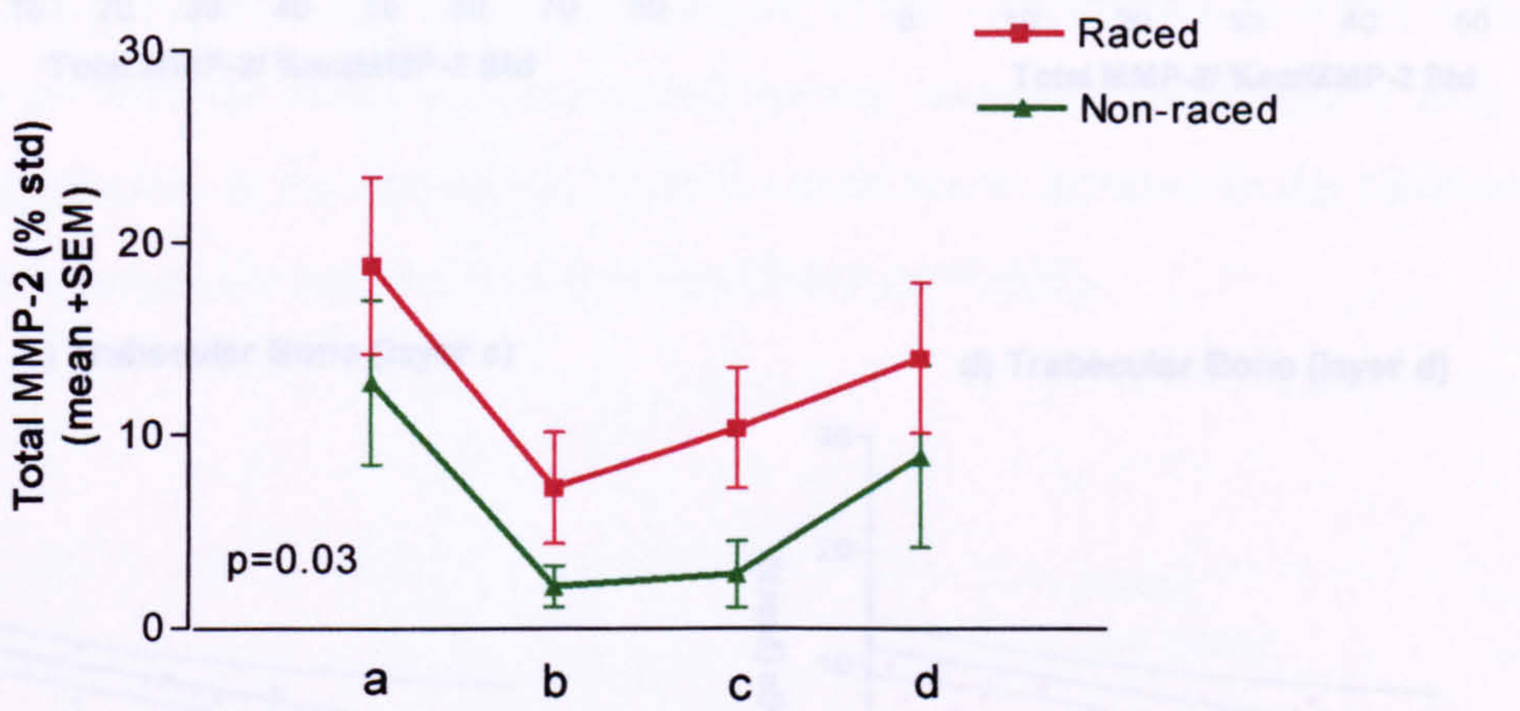


Figure 3.21: The total MMP-2 expression throughout the various layers of the Cr. The variation amongst the layers a to d was significant (p=0.03).

2) C3:

a) Right and left differences

There was a significant difference between the right (32.32+/-8.46) and left (20.6+/-7.34) total MMP-2 levels in layer a raced C3 (p=0.01) and between the right (15.53+/-5.41) and left (0.81+/-0.47) MMP-2 in layer a non-raced C3 (p=0.03) (see Appendix Three).

b) Correlation and covariation with age

Total MMP-2 had a significant correlation with age in all layers (a-d) of bone in the C3 (refer to Figure 3.22). There was additionally a significant covariation with age in layers c (p=0.01) and d (p= 0.02) but not in layers a (p= 0.12) and b (p=0.22).

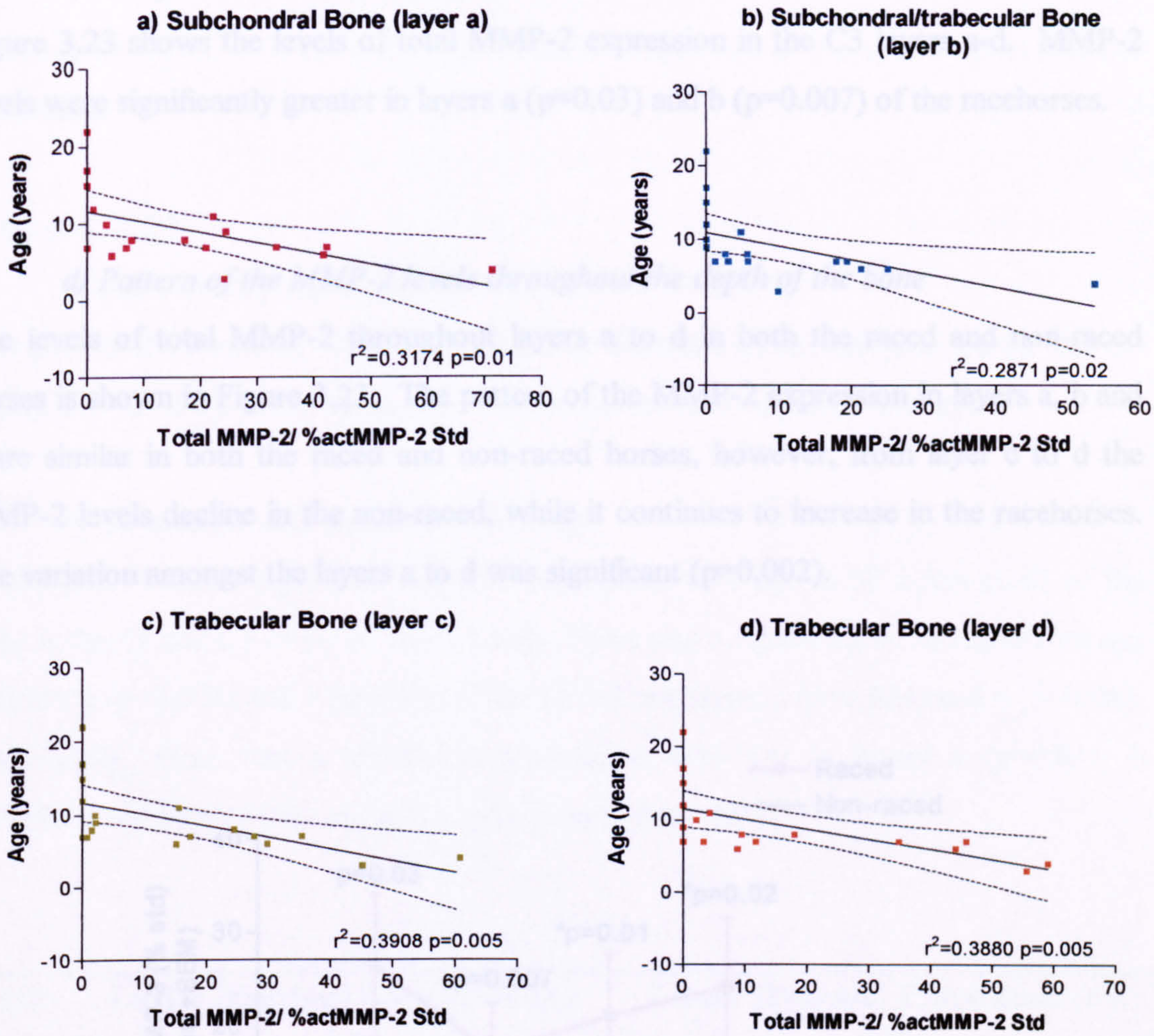


Figure 3.22: The correlation between age and the quantity of activated MMP-2 in the C3; a) layer a, b) layer b, c) layer c and d) layer d.

c) Comparisons between raced and non-raced horses

Figure 3.23 shows the levels of total MMP-2 expression in the C3 layers a-d. MMP-2 levels were significantly greater in layers a (p=0.03) and b (p=0.007) of the racehorses.

d) Pattern of the MMP-2 levels throughout the depth of the bone

The levels of total MMP-2 throughout layers a to d in both the raced and non-raced horses is shown in Figure 3.23. The pattern of the MMP-2 expression in layers a, b and c are similar in both the raced and non-raced horses, however, from layer c to d the MMP-2 levels decline in the non-raced, while it continues to increase in the racehorses. The variation amongst the layers a to d was significant (p=0.002).

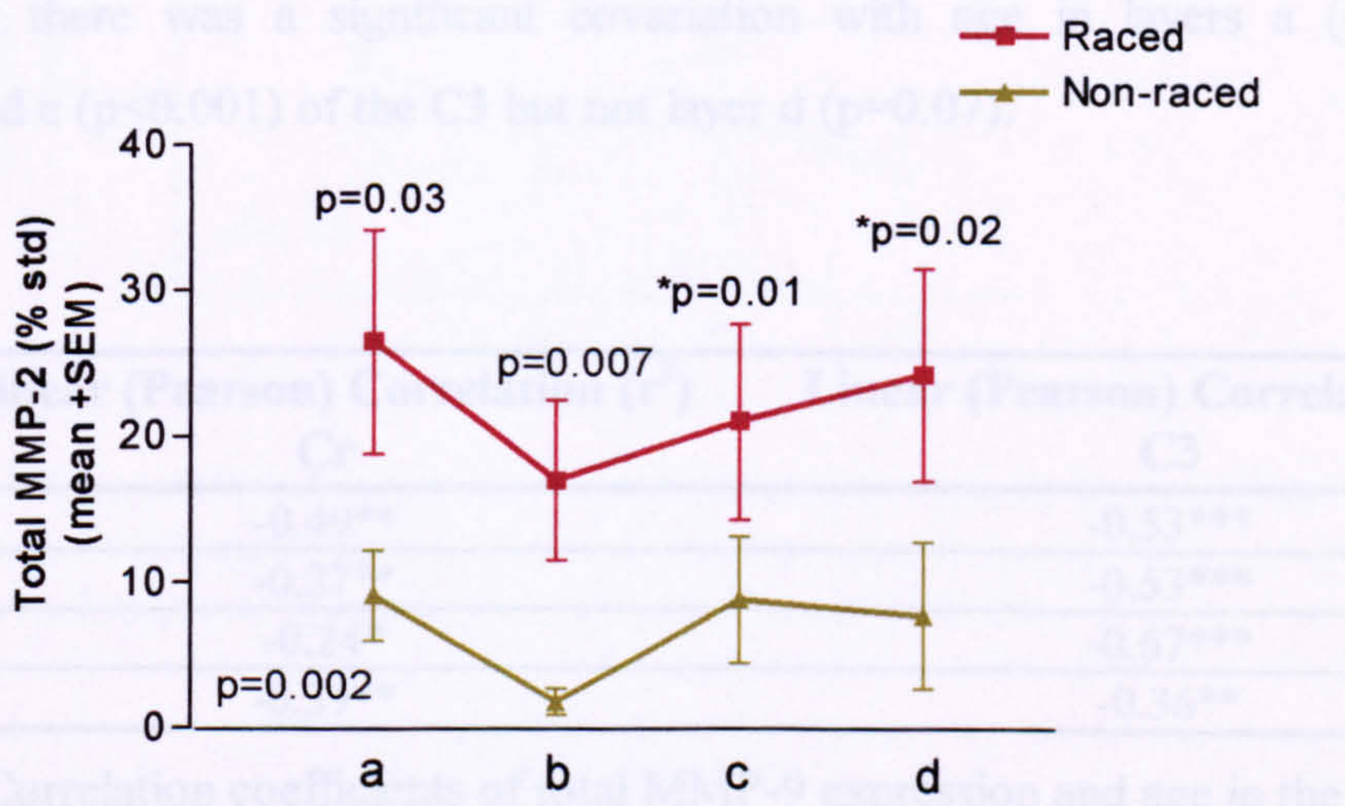


Figure 3.23: The total MMP-2 activity throughout the various layers of the C3. There was a significant difference between the raced and non-raced horses in layers a (p=0.03) and b (p=0.007). The variation amongst the layers a to d was significant (p=0.002).

3.1.3.3.4 Total MMP-9 Activity

As for section 3.1.3.3.3. the following results are expressed as the total level of MMP-9 expression.

1) Cr and 2) C3:

a) Right and left differences

The total MMP-9 activity was not significantly different in the right and left Cr and C3 (layers a to d) of either the raced or non-raced horses (see Appendix Three).

b) Correlation and covariation with age

Total MMP-9 had a significant negative correlation with age in all layers (a-d) of the bone in the Cr and C3 (refer to Table 3.16). There was a significant covariation with age in layers a ($p=0.001$) and b ($p=0.02$) of the Cr but not layers c ($p=0.054$) and d ($p=0.08$). Additionally, there was a significant covariation with age in layers a ($p=0.01$), b ($p=0.008$) and c ($p<0.001$) of the C3 but not layer d ($p=0.07$).

Layer	Linear (Pearson) Correlation (r^2) Cr	Linear (Pearson) Correlation (r^2) C3
a	-0.49**	-0.53***
b	-0.37**	-0.53***
c	-0.24*	-0.67***
d	-0.39**	-0.36**

Table 3.16: Correlation coefficients of total MMP-9 expression and age in the Cr and C3 layers a-d. Significant results; *($P<0.05$), **($P<0.01$), ***($P<0.001$).

c) Comparisons between raced and non-raced horses

There was no significant difference in the levels of total MMP-9 between the raced and non-raced horses in layers a-c of the Cr and the C3. However, there was a significant difference in layer d in both the Cr ($p=0.002$) (refer to Figure 3.24) and C3 ($p=0.01$) (refer to Figure 3.25).

d) Pattern of the MMP-9 levels throughout the depth of the bone

i) Cr:

The levels of total MMP-9 throughout layers a to d in both the raced and non-raced horses is shown in Figure 3.24. In both groups of horses, the MMP-9 levels are slightly elevated in layer a, decreasing from this point in layers b and c. In the racehorses, the levels of MMP-9 increase again from layer c to d, whereas in the non-raced the levels continue to decline. The variation amongst the layers a to d was significant (p=0.03).

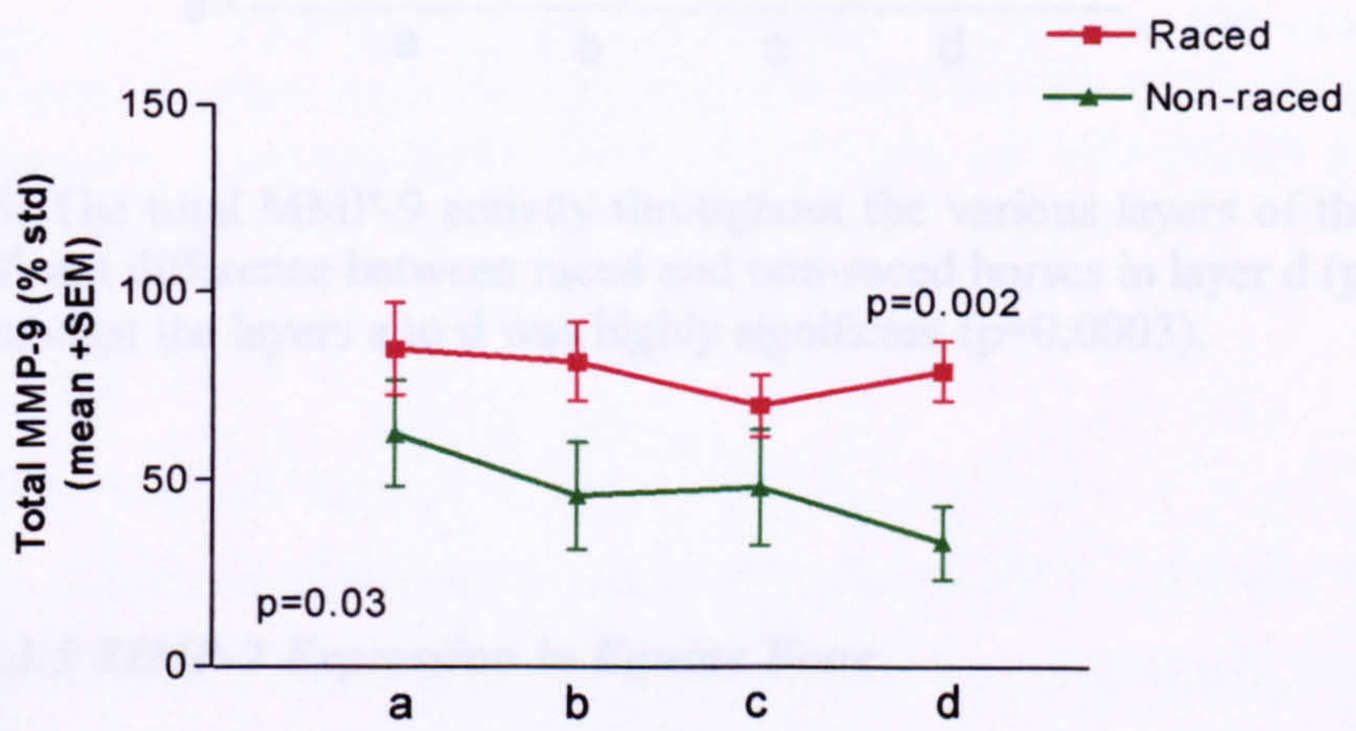


Figure 3.24: The total MMP-9 activity throughout the various layers of the Cr. There was a significant difference between raced and non-raced horses in layer d (p=0.002). The variation amongst the layers a to d was significant (p=0.03).

ii) C3:

The levels of total MMP-9 throughout layers a to d in both the raced and non-raced horses is shown in Figure 3.25. The pattern is similar in both groups of horses, decreasing from layer a to b and increasing from layers b to c. In the racehorses the MMP-9 levels continue to increase from layers c to d but the levels reach a plateau in the non-raced. The variation amongst the layers a to d was significant (p=0.0003).

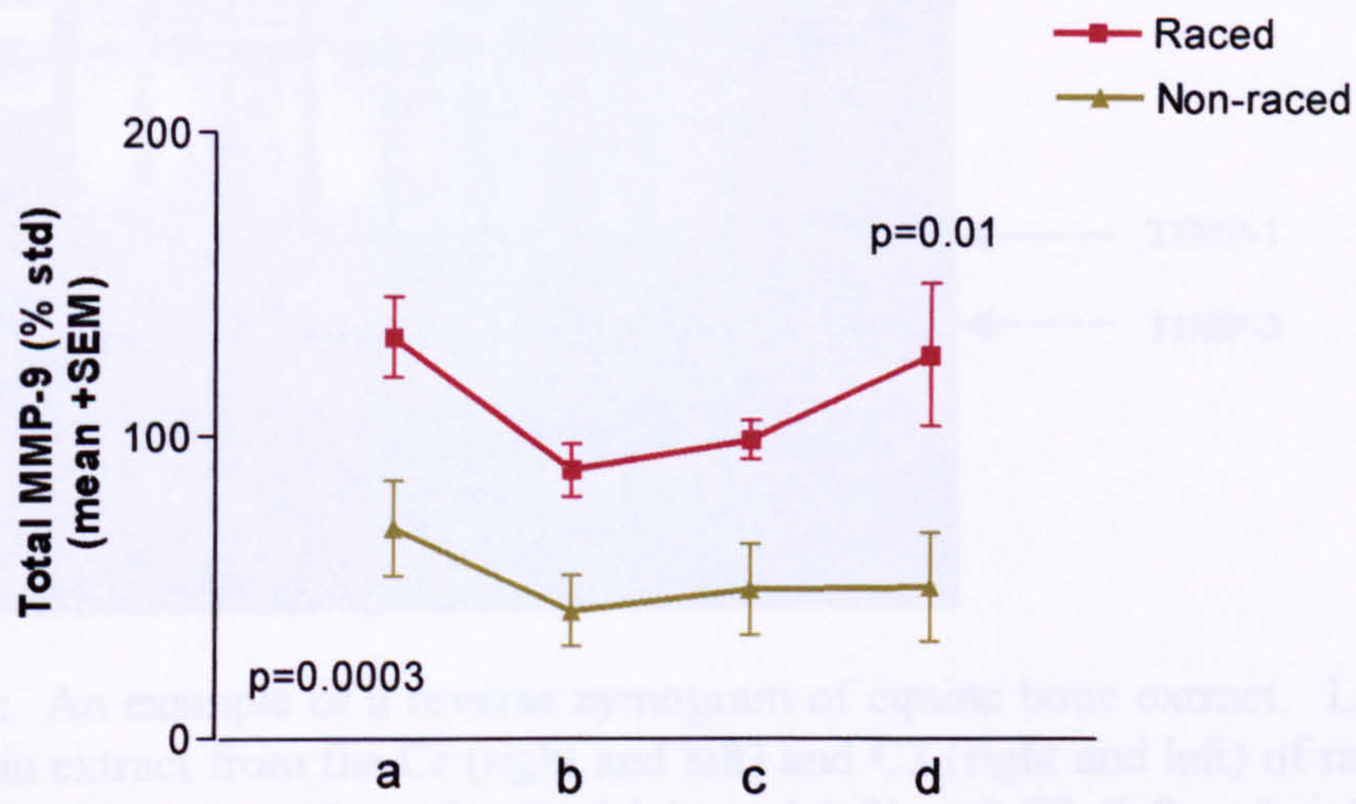


Figure 3.25: The total MMP-9 activity throughout the various layers of the C3. There was a significant difference between raced and non-raced horses in layer d (p=0.01). The variation amongst the layers a to d was highly significant (p=0.0003).

3.1.3.3.5 TIMP-2 Expression in Equine Bone

Figure 3.26 shows a typical reverse zymogram of equine bone extract. The molecular weights of TIMP-1 and TIMP-2 are 28 and 22kDa respectively. TIMP-2 levels were present in all samples analysed. No bands of 22kDa were present on the protein gels and the TIMP-2 of the samples migrated to the same point as the TIMP-2 standard on the zymogram, therefore this band represents levels of TIMP-2 in equine bone extract. TIMP-1 levels were present at far lower levels than TIMP-2, and hence it was difficult to quantify. TIMP-2 levels were therefore quantified whereas TIMP-1 levels were not.

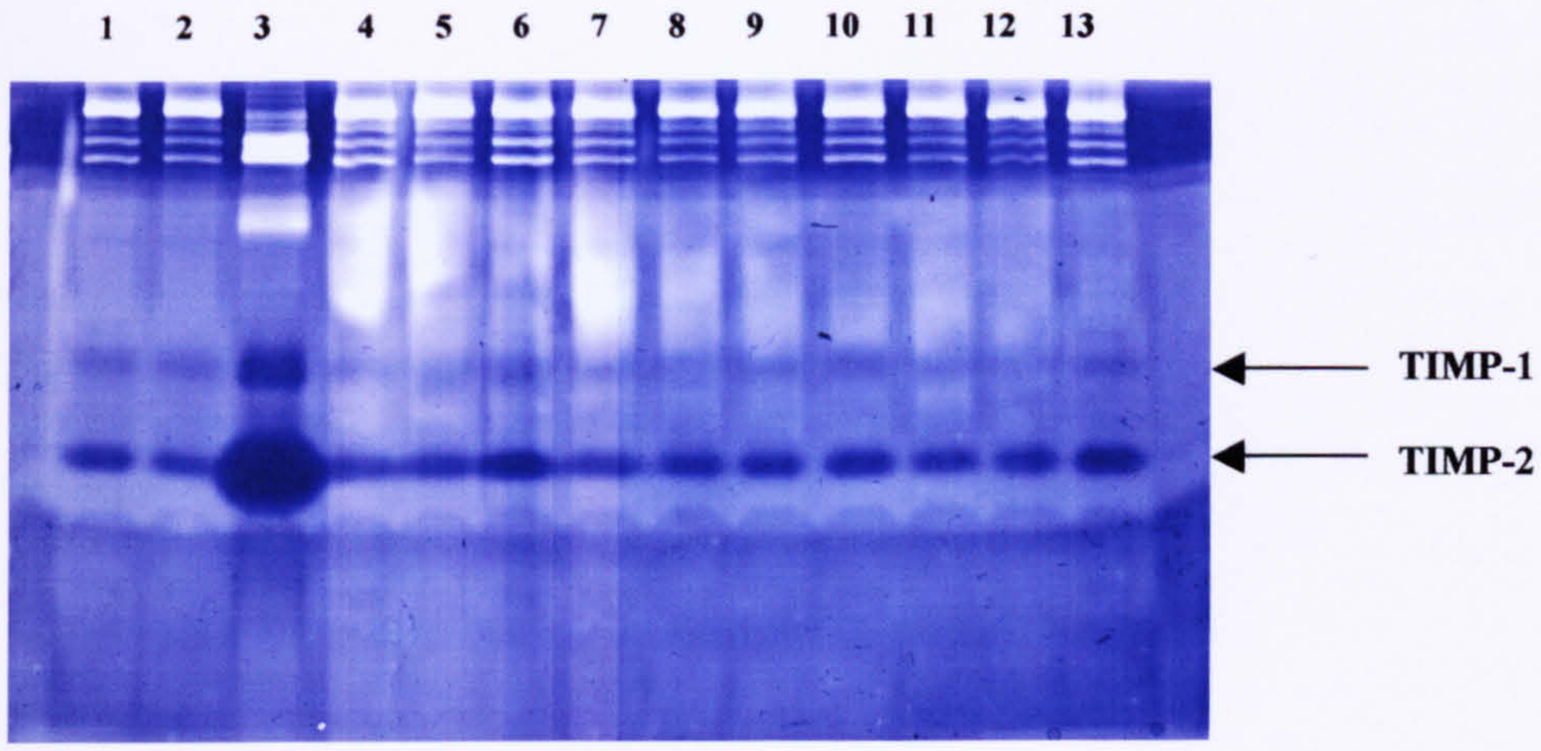


Figure 3.26: An example of a reverse zymogram of equine bone extract. Lanes 1, 2, 4 and 5 contain extract from the Cr (right and left) and C3 (right and left) of raced layer b. Lanes 6-9 contain extract from the Cr (right and left) and C3 (left and right) of raced layer c. Lanes 10-13 contain extract from the Cr (right and left) and C3 (right and left) of raced layer d. Lane 3 contains the TIMP-2 standard.

1) Cr and 2) C3:

a) Right and left differences

There was a significant difference between the right (21.34+/-5.87) and left (16.67+/-4.83) TIMP-2 levels in the Cr of the non-racehorses in layer d ($p=0.04$), but this was the only significant difference observed in both the Cr and C3 (see Appendix Three).

b) Correlation and covariation with age

TIMP-2 levels did not correlate with age in any layer of the Cr and C3 (refer to Table 3.17).

Layer	Linear (Pearson) Correlation (r^2)	P Value
Cr		
a	-0.02	0.68
b	-0.20	0.26
c	-0.01	0.77
d	-0.13	0.36
C3		
a	-0.0005	0.95
b	-0.18	0.22
c	-0.02	0.68
d	-0.14	0.31

Table 3.17: Correlation coefficients of the cross-link content and age in the Cr and C3 layers a-d.

c) Comparisons between raced and non-raced horses

There was no significant difference in the TIMP-2 levels in any layer of the Cr and C3 (refer to Figures 3.27 and 3.28 respectively).

d) Pattern of the TIMP activity throughout the depth of the bone

i) Cr

The pattern of TIMP-2 levels throughout the depth of the Cr are shown in Figure 3.27. The TIMP-2 activity in both the raced and non-raced horses is variable, with no defined pattern. TIMP-2 decreases from layer a to b in both the raced and non-raced, decreasing further in the non-raced but increasing in the raced from layer b to c. From this point (layer c to d) the TIMP-2 activity decreases in the raced and increases in the non-raced. There was no significant variation amongst the layers.

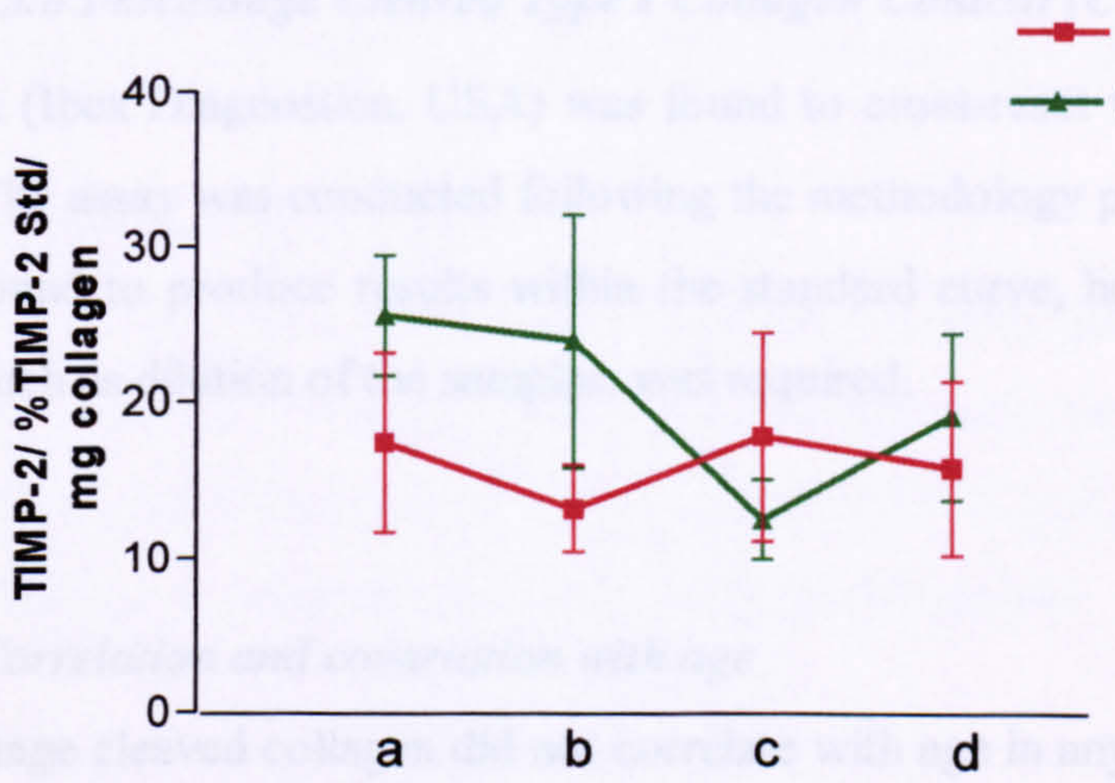


Figure 3.27: The TIMP-2 activity in layers a to d of the Cr.

ii) C3

Figure 3.28 shows the pattern of the TIMP-2 levels in layers a to d of the C3. Similar to the Cr, the pattern in both the raced and non-raced is varied. Again, the TIMP-2 activity decreases from layer a to b in both groups of horses, but conversely to the Cr, the activity increases from layer b to c in the non-raced and decreases in the raced. The TIMP-2 activity then decreases in the non-raced and increases in the raced from layer c to d. There was no significant variation amongst the layers.

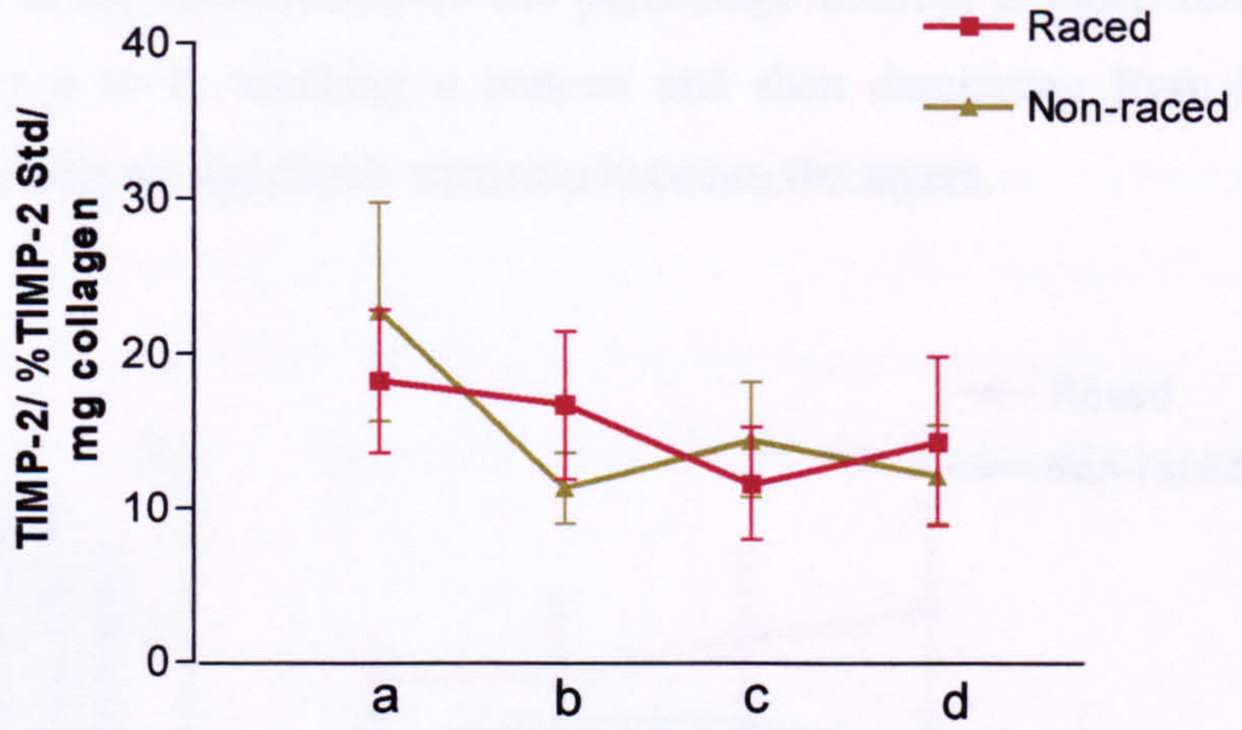


Figure 3.28: The TIMP-2 activity in layers a to d in the C3.

3.1.3.3.6 Percentage Cleaved Type I Collagen Content (COL2-3/4C$_{short}$ epitope)

The ELISA (Ibex Diagnostica, USA) was found to cross-react with equine type I bone collagen. The assay was conducted following the methodology provided by the supplier. This was found to produce results within the standard curve, hence no modification to the assay, such as dilution of the samples, was required.

1) Cr:

a) Correlation and covariation with age

The percentage cleaved collagen did not correlate with age in any layer of the Cr; layer a (r^2= 0.06, p=0.37), b (r^2= 0.008, p=0.73), c (r^2= 0.05, p=0.41), and d (r^2= 0.04, p=0.51).

b) Comparisons between raced and non-raced horses

There was no significant difference in the percentage cleaved collagen between the raced and non-raced horses, although there was a trend for an increased percentage in the raced in all layers (refer to Figure 3.29).

c) Pattern of the % cleaved collagen throughout the depth of the bone

The pattern of the percentage cleaved collagen content throughout the depth of the Cr is shown in Figure 3.29. In the racehorses, the percentage content increases from layers a

to d, whereas in the non-racehorses the percentage content is more varied, increasing between layers a to b, reaching a plateau and then decreasing from layers c to d, however, there was no significant variation between the layers.

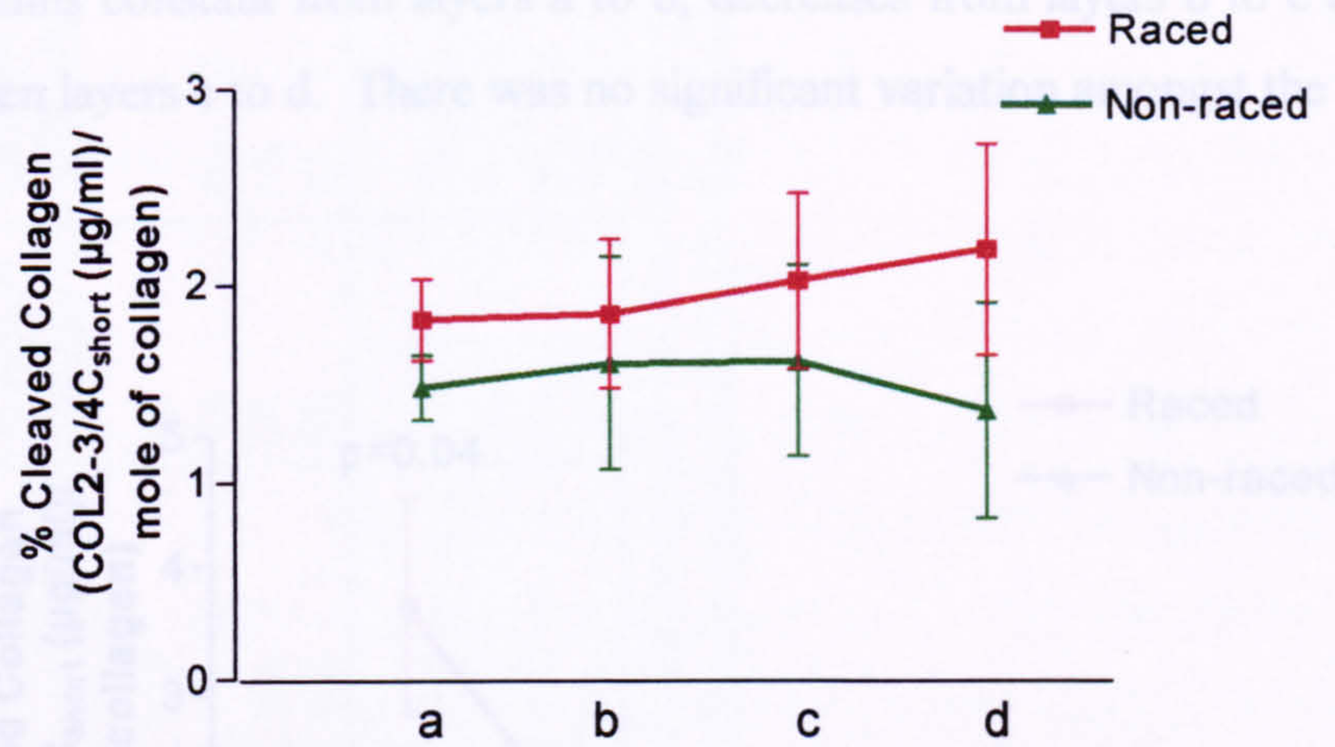


Figure 3.29: The % cleaved collagen content throughout the various layer of the Cr.

d) Correlation with MMP-2

There was no relationship between the total MMP-2 activity and the percentage cleaved collagen or the amount of COL2-3/4C$_{short}$ neoepitope expressed in µg/ml in both the raced and non-raced horses in layers b, c and d. MMP-2 had a significant covariation with age in layer a, so this layer was not included in the analysis.

2) C3:

a) Correlation and covariation with age

There was no significant correlation in the percentage cleaved collagen with age in any layer of the C3; layer a (r^2= 0.13, p=0.15), b (r^2= 0.001, p=0.88), c (r^2= 0.06, p=0.32) and d (r^2= 0.14, p=0.15).

b) Comparisons between raced and non-raced horses

There was a significant difference between the raced and non-raced horses in layer a (p=0.04) only, the % cleaved collagen being significantly greater in the racehorses (refer to Figure 3.30).

c) Pattern of the % cleaved collagen throughout the depth of the bone

Figure 3.30 shows the pattern of the percentage cleaved collagen throughout the depth of the C3. In the racehorses, the percentage content dramatically decreases from layer a to b and then increases slightly from layers b to d. In the non-racehorses, the percentage content remains constant from layers a to b, decreases from layers b to c and increases again between layers c to d. There was no significant variation amongst the layers.

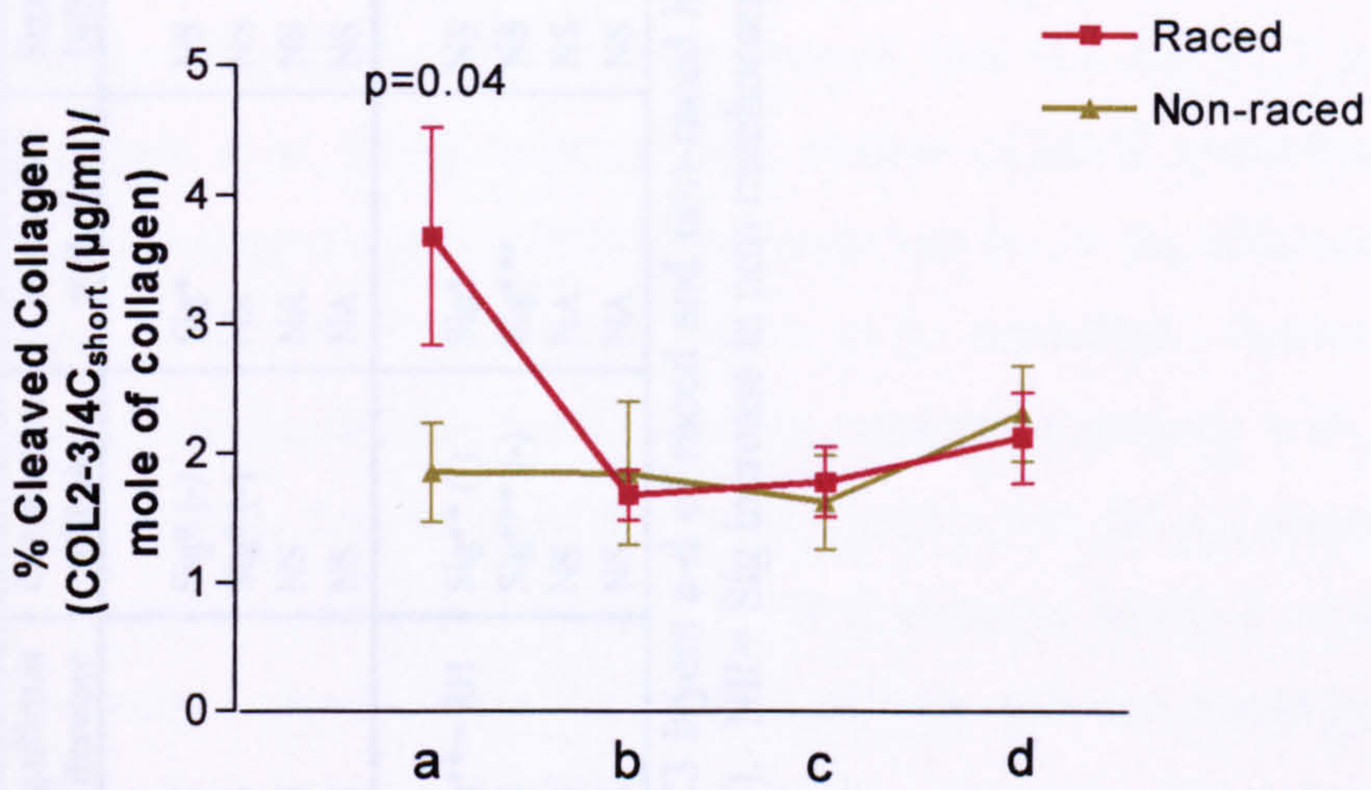


Figure 3.30: The % cleaved collagen content throughout the various layer of the C3. There was a significant difference between raced and non-raced in layer a (p=0.04).

d) Correlation with MMP-2

No relationship was found between the total MMP-2 activity and the percentage cleaved collagen or the amount of COL2-3/4C$_{short}$ neoepitope expressed in µg/ml in the raced and non-raced horses in layers a and b. MMP-2 had a significant covariation with age in layers c and d, so these layers were not included in the analysis.

Marker of Degradation	Layer a			Layer b			Layer c			Layer d		
	Correlation with Age	Covariation with Age	Significant Difference	Correlation with Age	Covariation with Age	Significant Difference	Correlation with Age	Covariation with Age	Significant Difference	Correlation with Age	Covariation with Age	Significant Difference
Cr												
MMP-2	Sig*** (-)	Sig**	NS	Sig** (-)	NS	NS	Sig* (-)	Sig*	NS	NS	NA	NS
MMP-9	Sig*** (-)	Sig***	NS	Sig** (-)	Sig*	NS	Sig* (-)	NS	NS	Sig** (-)	NS	Sig**= RH
TIMP-2	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
COL2-3/4C	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
C3												
MMP-2	Sig* (-)	NS	Sig*= RH	Sig* (-)	NS	Sig**= RH	Sig** (-)	Sig*	NS	Sig** (-)	Sig*	NS
MMP-9	Sig*** (-)	Sig*	NS	Sig*** (-)	Sig**	NS	Sig*** (-)	Sig***	NS	Sig** (-)	NS	Sig*= RH
TIMP-2	NS	NA	NS	NS	NA	NS	NS	NA	NS	NS	NA	NS
COL2-3/4C	NS	NA	Sig*= RH	NS	NA	NS	NS	NA	NS	NS	NA	NS

Table 3.18: Summary of the markers of bone degradation in the Cr and C3 layers a-d of raced and non-raced horses. NS= Not significant. NA= Not applicable. Sig= Significant results= *($P<0.05$), **($P<0.01$), ***($P<0.001$). NR= Sig increase in non-racehorses. RH= Sig increase in racehorses. (-)= Negative correlation with age.

3.1.3.4 Discussion

Relationships with Age

The relationships between age and collagen degradation are clearly shown in this study, especially with regard to the expression of MMP-2 and-9, which in most layers of the Cr and C3 levels decline with age. This finding is not unexpected since in younger animals collagen turnover will have to be maintained at a substantially higher level than older animals to allow for growth and modelling. MMP expression has been previously shown to have a negative correlation with age in the synovial fluid from the MCP joint of horses (Brama, TeKoppele et al. 1998), however, the method of MMP quantification used in this study only represented a general MMP substrate and hence the influence of age on a specific member of the MMP family was unable to be quantified. Recently, however, stromelysin (MMP-3) levels have been shown to correlate negatively with age (Brama, TeKoppele et al. 2000). Interestingly, the TIMP-2 expression did not correlate with age in any layer of the Cr and C3 and since TIMP-2 controls MMP-2 activity in a 1:1 stoichiometric ratio, a correlation in the MMP-2 activity with age would be expected to result in a similar correlation in the TIMP-2 activity. However, this may be due to the resulting effects of high-intensity exercise on the imbalance of MMP and TIMP activity.

The percentage cleaved collagen, determined from the amount of COL2-3/4C_{short} neoepitope, did not correlate with age in any layers of the Cr and C3. This lack of correlation is less easy to explain, especially in light of the MMP-2 and –9 data, since an increase in the percentage cleaved collagen reflects cleavage of type I collagen by the collagenases (members of the MMP family) and thus would be expected to decline with age as for the MMP-2 and –9 data. However, in the only study to date examining the influence of age on the amount of cleaved type II collagen (determined from expression of the COL2-3/4C_{short} neoepitope) by Aurich *et al.*, (2002) the amount of cleaved collagen in the ankle of normal human cartilage did not correlate with age either (Aurich, Poole et al. 2002), thus supporting the data in this study (although it is important to note that the study by Aurich *et al.*, was undertaken on 'normal' human individuals).

The Effects of Exercise

Racing and race-training does appear to be inducing an increased level of collagen degradation in all layers of the Cr and C3, although, the levels of collagen degradation appear to be less in the Cr than the C3, which may be explained by the confounding influence of age on the markers of collagen degradation in the Cr.

The increased total (both pro and active forms) MMP-2 and-9 activity in the C3 of racehorses is indicative of remodelling of the collagen matrix (Creemers, Jansen et al. 1998; Clegg and Carter 1999; Kerkvliet, Docherty et al. 1999) and the up regulation of MMP-2 and-9 has been documented in the synovial fluid and cartilage of OA and OC equine joints (Clegg, Coughlan et al. 1997; Clegg and Carter 1999; Trumble, Trotter et al. 2001; Al-Hizab, Clegg et al. 2002).

Interestingly, total MMP-9 activity is shown to be up regulated in the Cr and C3 of the racehorses in layer d only. Its significant activation in this layer is not unexpected since this is an inflammatory mediated MMP being released by neutrophils and monocytes i.e. marrow-derived cells, and its role in inflammation has been extensively documented (Ahrens, Koch et al. 1996; Clegg, Coughlan et al. 1997; Tarlton, Vickery et al. 1997; Clegg and Carter 1999; Tarlton, Whiting et al. 2000; Trumble, Trotter et al. 2001). Furthermore, throughout the depth of bone in the Cr and C3 the activity of MMP-9 (and to a lesser extent MMP-2) increases in the racehorse from layer c to d to values similar to that seen in the cortical bone, but this does not occur in the non-raced bone. This increase in MMP-9 activity may be a response of the 'inflammatory' cells to the increased mechanical load being placed on the racehorse bone.

The natural inhibitor of MMPs, TIMP-2, was found not to be significantly elevated in any layers of the Cr and C3 of the raced compared to the non-raced horses. This lack of TIMP-2 activation in the raced C3, despite the up-regulation of MMP-2, indicates that there is an imbalance of MMP and TIMP activity with high-intensity exercise, resulting in the increased degradation of the collagen molecules as is seen in many diseased tissues (Dean, Azzo et al. 1987; Lohmander, Hoerrner et al. 1993; Huang, Ding et al. 1997; Clegg, Coughlan et al. 1998; St'ovickova, Hulejova et al. 2002).

As expected from the MMP data, the percentage cleaved collagen was also found to be elevated in the raced Cr and C3, further indicating increased collagen degradation with high-intensity exercise. This effect of exercise on collagen degradation has also been demonstrated in the serum of exercised foals by Billinghurst *et al.*, (2003), in particular it was shown that type I collagen degradation was significantly increased in foals with increased levels of exercise compared to normal pasture exercise during the first five months of life (Billinghurst, Knowlton et al. 2003).

The gelatinases MMP-2 and-9, are primarily capable of degrading collagen that has been previously cleaved by the collagenases, but a possible role of MMP-2 to cleave native collagen has been postulated (Aimes and Quigley 1995; Patterson, Atkinson et al. 2001). The expression of COL2-3/4C_{short} neoepitope is a consequence of collagenase activity, and since a possible role of MMP-2 to exhibit collagenolytic activity has been suggested, a correlation between the amount of MMP-2 activity and the percentage cleaved collagen might be expected. However, in this study this correlation was not found to exist and as such may question the collagenolytic role of MMP-2. However, no firm conclusion can be drawn from these data and hence it can only be suggested that quantification of the COL2-3/4C_{short} neoepitope is not an ideal marker of MMP-2 activity.

Throughout the depth of the bone, in both the raced and non-raced horses, the levels of MMP-2 and –9, and the percentage cleaved collagen, are relatively similar in layers a to c, with levels being high in layer a, declining from layers a to b, and increasing from layers b to c. However, the levels differ between the two groups of horses from layers c to d, most notably within the racehorses levels increase, often to levels comparable to layer a, suggesting the mechanical loads experienced during racing and race-training are resulting in increased levels of collagen degradation in the deeper regions of the bone. Degradation of the collagen matrix within the trabecular bone may result in a mechanically weaker tissue, which may ultimately lead to failure if the level of exercise intensity were to continue.

3.1.4 The Thermal Properties of Collagen

3.1.4.1 Introduction

The mechanical properties of bone are governed by several factors, including the ultrastructural and molecular organisation of the collagen fibrils. The collagen cross-links are known to contribute to the mechanical properties of bone (Knott, Whitehead et al. 1995; Liu, Yang et al. 1995; Oxlund, Barckman et al. 1995), but the mechanical strength is also governed by the integrity of the collagen molecules within the fibrils. This can be assessed by measuring the thermal properties of the bone collagen using differential scanning calorimetry (DSC).

DSC allows the study of the thermal denaturation of proteins and involves simultaneously raising the temperatures of a sample and reference at a predetermined rate while maintaining identical temperatures in the sample and reference. Denaturation of the sample results in an increase in the power required to raise the temperature of the sample over that required to raise the temperature of the reference by the same amount. This power difference plotted against temperature gives the thermogram, and the point at which denaturation of the protein occurs is marked by a peak, or endotherm, in the thermogram. The thermal transition observed by DSC is the helix-coil transition, the transition from triple helix to more or less random coil (Privalov 1982). This is a molecular event, and thus sensitive to any change in the state of the molecule such as, changes in molecular composition, post-translational modifications, molecular organization and cross-linking, and chemical environment, such as mineralisation (Bailey, Sims et al. 1993; Miles, Wardale et al. 1994; Miles, Burjanadze et al. 1995). DSC analysis of the collagen can therefore provide substantial information concerning the state of the collagen in the tissue.

DSC Terminology

Enthalpy of Denaturation: The enthalpy of denaturation of collagen is the energy required to reduce the native collagen triple helix to random coil and is termed ΔH. This is represented by the area under the peak and is usually expressed in Jg^{-1} collagen.

T_{max}: T_{max} is the temperature at which the peak apex is reached.

The enthalpy of denaturation and the T_{max}, are sensitive to different molecular properties. T_{max} is governed by the rate of helix unfolding (Miles 1993; Miles, Burjanadze et al. 1995; Miles and Bailey 2001; Miles and Bailey 2004). This rate is markedly slowed and T_{max} increased by the close proximity of other collagen molecules. Thus a dilute solution of mammalian collagen yields T_{max} in the region of 40°C, whereas the same molecules closely surrounded by other similar molecules in the fibres of a tendon at physiological hydrations yields a higher T_{max} (60-70°C). The same molecules even more closely packed in dehydrated fibres yield even higher temperatures (up to 210°C for the very driest fibres) (Miles and Ghelashvili 1999). The stabilisation induced by this mechanism is dependent on the amount of space around the molecule, which is known as the 'polymer in a box' mechanism of stabilisation (Miles and Burjanadze 2001). Imagine a folded polymer enclosed in a box. This polymer is more stable than a similar molecule that is not enclosed. This is because the molecule needs space to unfold. The smaller the box is, the slower the unfolding, and the greater the stabilisation.

The enthalpy of the transition is however, governed by the energy of the bonds holding the triple helix together. The precise nature of these bonds is still subject to debate, although water-collagen hydrogen bonds are thought to be important by some, but not by others (Privalov 1982; Engel and Prockop 1998; Miles and Burjanadze 2001). What is known however is that the enthalpy of denaturation of mammalian collagen in fibres is practically constant at about 70J/g over a wide range of hydrations (above about 6 molecules of water per Gly-X-Y triplet) but diminishes rapidly to about 10J/g at low hydrations (below 1 molecule of water per Gly-X-Y triplet) (Miles and Ghelashvili 1999). The residual 10J/g is thought to be due to the direct hydrogen bond connecting each glycine NH on one chain to the backbone C=O of a 2nd position residue on an adjacent chain.

Current Research

To date, limited information exists regarding the thermal characteristics of bone collagen, especially with regards to the effect of exercise on these thermal characteristics.

Previous studies of bovine and rat bone collagen have found that the denaturation temperature (T_{max}) of bone is high compared with that of soft collagenous tissues, probably due to mineralisation and dehydration of the fibres (Calafiori, Imbrogno et al. 1993; Kronick and Cooke 1996). The presence of two denaturation events in mineralised tissue has also been observed (Calafiori, Imbrogno et al. 1993; Kronick and Cooke 1996; Knott, Tarlton et al. 1997). The characteristics of equine bone collagen are unknown.

In addition, the relationship with age and the thermal properties of the collagen, especially with regard to bone has received scant attention. Flandin *et al.*, (1984) used DSC to analyse the age-related changes in the thermal stability of rat skin collagen. The endotherm consisted of one large and two smaller peaks either side. Between 2 and 20 months the first smaller peak diminished and the third peak increased, which correlated with a decrease in HLNL. They concluded that the replacement of these immature collagen cross-links with mature cross-links accounted for the thermal stabilisation of the collagen with age. The total denaturation enthalpy did not vary with age (Flandin, Buffevant et al. 1984). More recently, Knott *et al.*, (1997) found that the two peaks (one sharp peak and a second broader peak) observed in the distal (non-calcifying) and the proximal (calcifying) region of the mineralising turkey leg tendon (MTLT), changed with increasing age with the second broader peak becoming more prominent, at the expense of the first peak, with age. Additionally, the T_{max} of the main peak (peak one) in both regions of the MTLT was found to increase with age. This change in the thermal stability of the collagen was comparable to the decrease in immature cross-links.

Knott *et al.*, (1997) also found that the sum of the enthalpies of peaks one and two of the proximal tendon decreased with age and with mineralisation. This decrease in enthalpy was not evident in the distal region (Knott, Tarlton et al. 1997), suggesting that the mineralisation process (not age) may be affecting the enthalpy of denaturation, possibly by disrupting the hydrogen bonds stabilising the collagen triple helix.

The purpose of this part of the study was to quantify the thermal characteristics of the collagen in the Cr and C3 of raced and non-raced horses.

3.1.4.2 Materials and Methods

3.1.4.2.1 Equine Samples

Table 3.19 shows the horses used to quantify the thermal properties of the collagen. The left and right of the Cr and C3 (layers a to d) were analysed.

Raced	Age	Non-raced	Age
R1	7	7	10
R5	6	**8**	11
R6	7	**9**	8
R7	7	**10**	NA
R8	7	**11**	3
R9	6		

Table 3.19: The horses used to quantify the thermal properties of the collagen and their ages (NA= Not Known).

3.1.4.2.2 Tissue Preparation

The pulverised bone was decalcified in 0.5M EDTA (tetrasodium salt) pH 7.5 for 2 days (at a concentration of 10mg (ww)/5ml EDTA) with a change of EDTA after the 1st day, and subsequently washed for 1hr in 10ml 2M $CaCl_2$ to remove any residual EDTA and then washed thoroughly with dH_2O (x2). The reasons for this protocol will be discussed later within this chapter (refer to section 3.2). The demineralised bone was frozen at –20°C until required.

3.1.4.2.3 Differential Scanning Calorimetry (DSC)

Approximately 5-15mg of decalcified bone was sealed in an aluminium pan (Perkin-Elmer) with a drop of dH_2O, weighed and then scanned from 5 to 130°C at 10°C per minute in a Perkin-Elmer DSC calorimeter (Beakonsfield Bucks, UK) fitted with intracooler and computer controller. An empty pan was used as a reference. After DSC analysis, holes were punched in the pans and the samples were dried overnight in a 105°C oven to obtain the dry weight of the tissue. The aluminium pans were subsequently hydrolysed in 5ml 6N HCL overnight and assayed for hydroxyproline as previously described, to determine the collagen content.

3.1.4.2.4 Statistical Analysis

Statistical analysis was performed using SPSS v11.5 (SPSS Inc. USA) and Graphpad Prism v3.0 (Graphpad Software Inc. USA), as detailed in Chapter Two section 2.2.1.5.

3.1.4.3 Results

A typical DSC thermogram of demineralised equine bone collagen is shown in Figure 3.31a (the thermograms do not differ between the cortical and trabecular layers of bone). Figure 3.31a illustrates that the equine bone endotherm is multicomponent being comprised of one relatively sharp transition, with a denaturation temperature ranging from 55-60°C (1st peak), superimposed on a much broader transition with a denaturation temperature ranging from 80-100°C (2nd peak). In contrast, fibres of type I collagen from the tail of young rats, possess only a single sharp denaturation temperature at about 60-70°C (refer to Figure 3.31b) (Miles, Burjanadze et al. 1995).

a) Right and left differences

There was a significant difference in the whole peak enthalpy of denaturation between right (53.37+/-5.39) and left (66.08+/-3.31) in layer b ($p= 0.008$), and between right (82.1+/-6.96) and left (55.46+/-3.49) in layer c ($p= 0.03$) of the raced Cr. There were no significant differences between the right and left in either the non-raced, the C3 or the T_{max} (refer to Appendix Three).

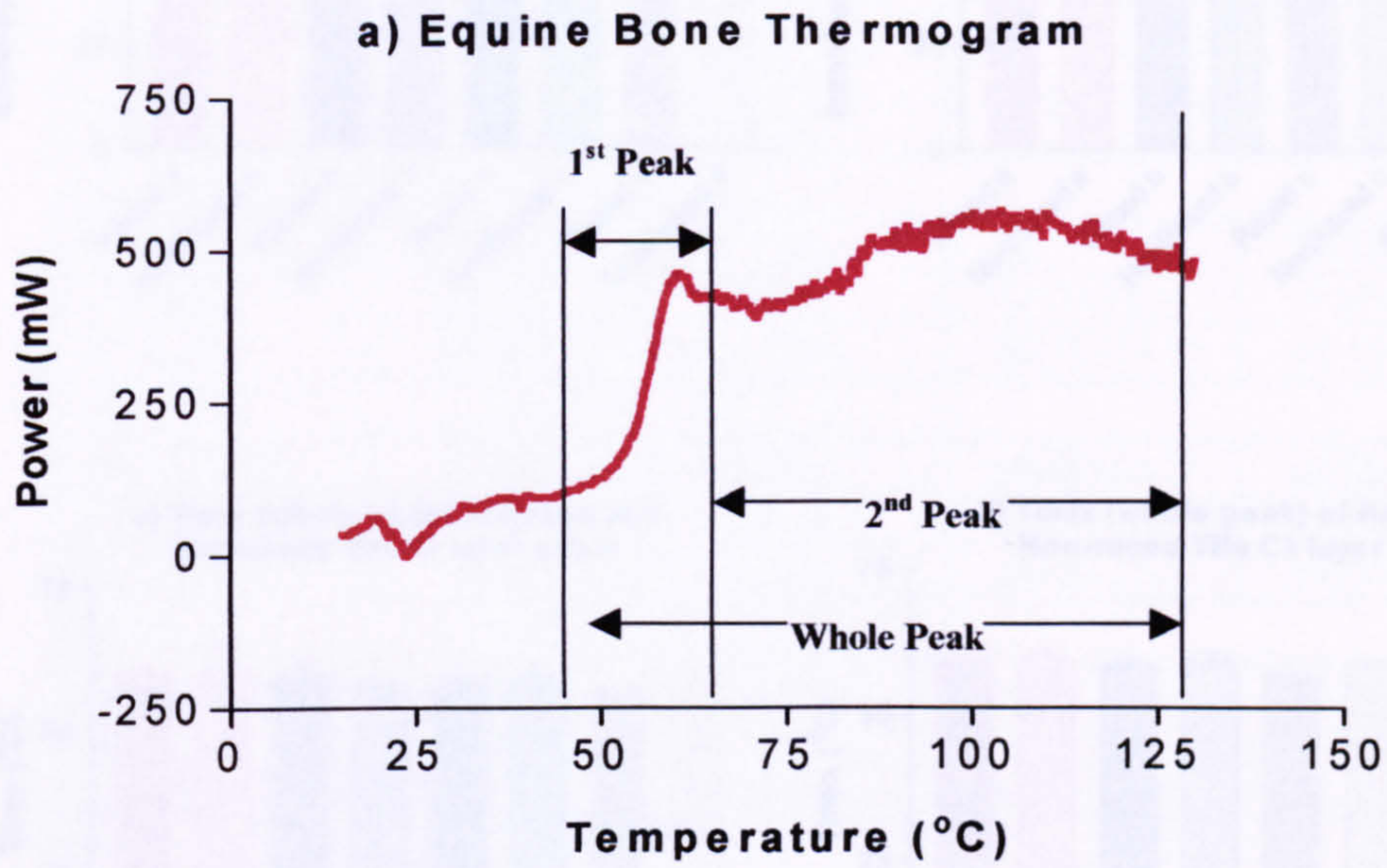
b) Comparisons between raced and non-raced horses

Table 3.20 shows a summary of the enthalpy results and Table 3.21 shows a summary of the T_{max} results of the whole peak, the 1st peak and the 2nd peak of the equine bone collagen. In general, there was a trend for a greater enthalpy of denaturation in the Cr and C3 of non-racehorses compared to racehorses (refer to Figure 3.32a and b), but there was no difference in the T_{max} (refer to Figure 3.32c and d).

c) Correlation with age

The relationships with age and the enthalpy, and T_{max}, were varied and complex between the layers and the peaks, with some positive and negative correlations with age being

apparent (refer to Tables 3.20 and 3.21). However, there was no change in the shape of the peaks with age (refer to Figure 3.32e).



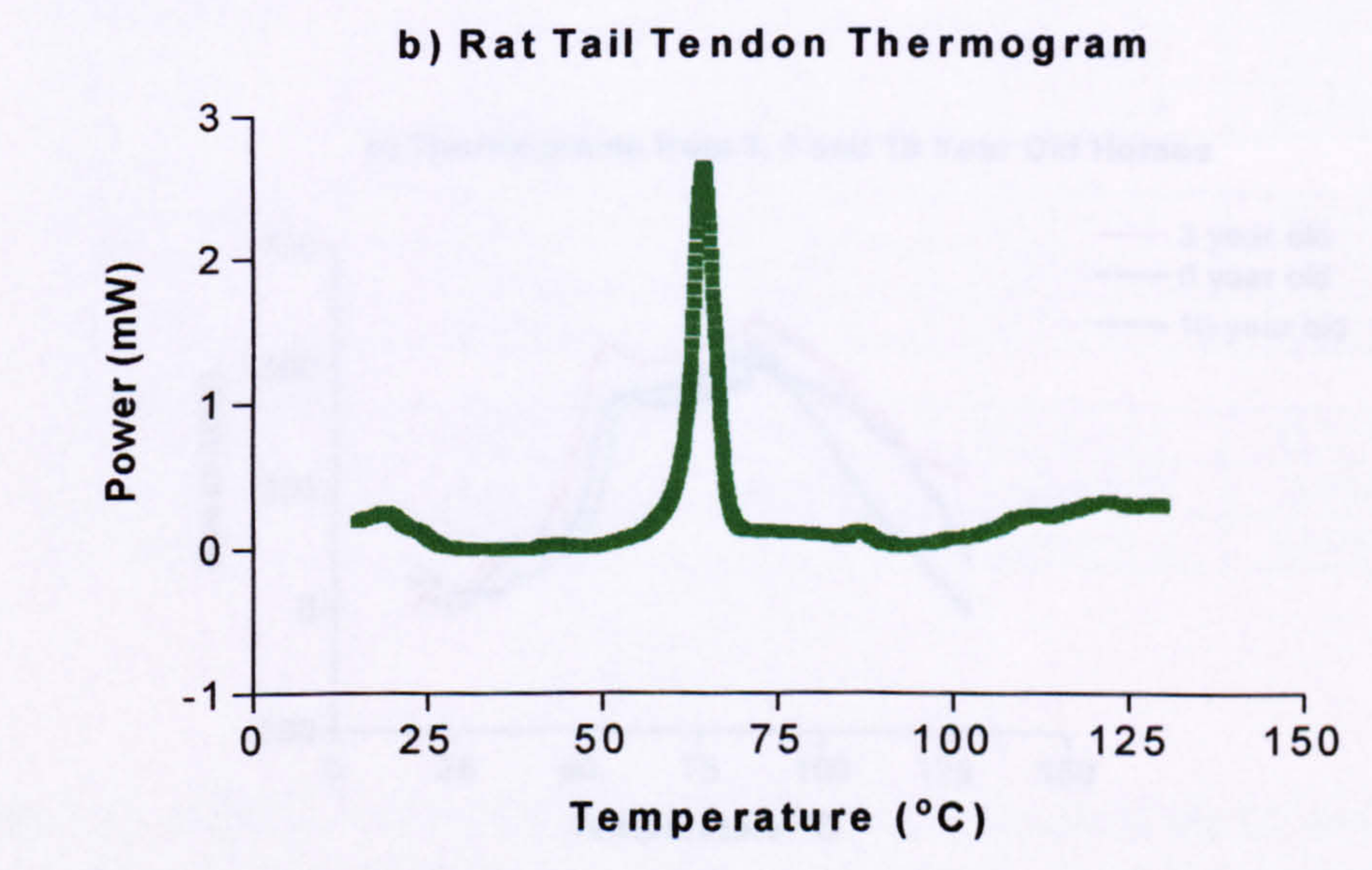


Figure 3.31: Typical differential scanning calorimetry thermograms of a) equine bone and b) rat tail tendon.

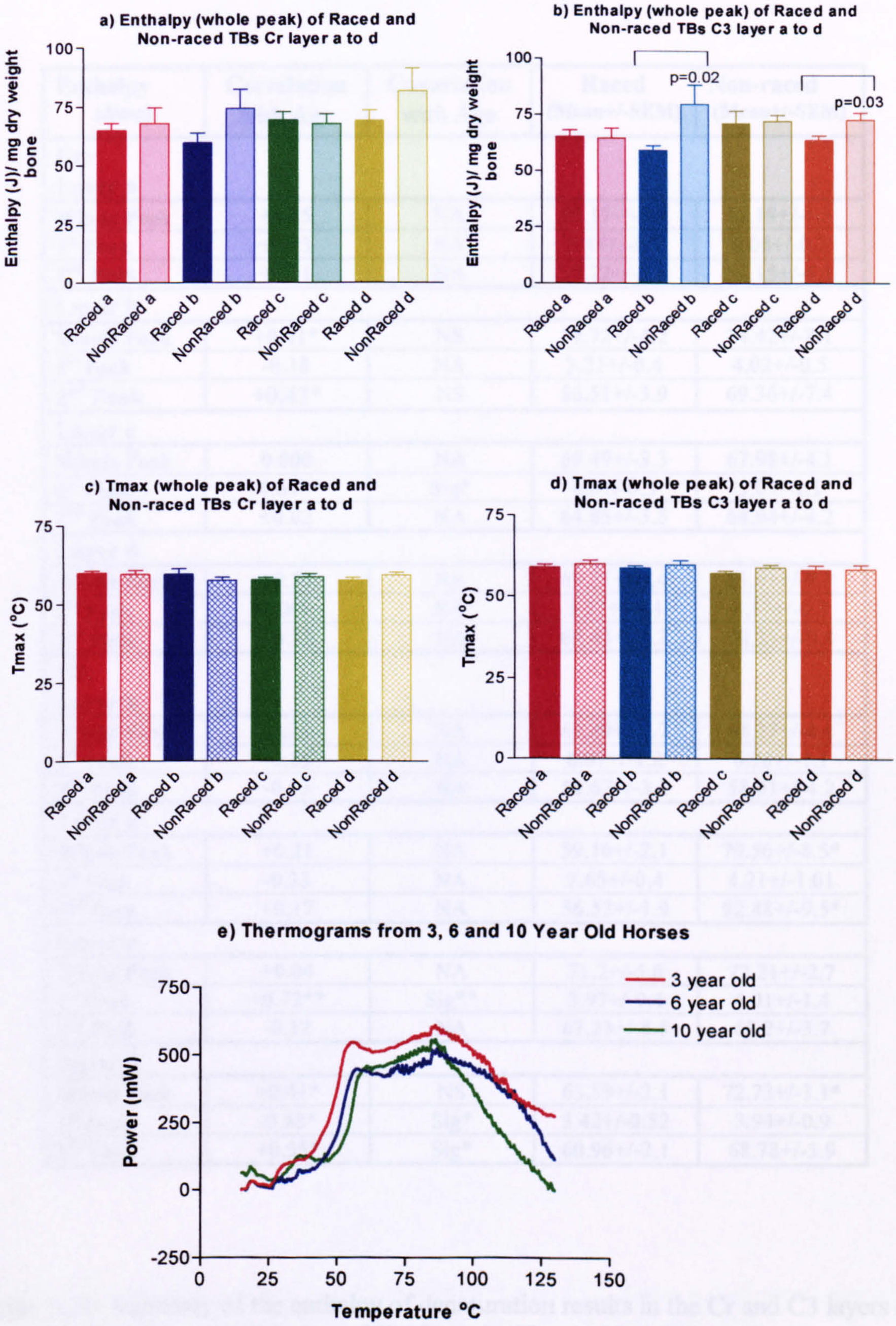


Figure 3.32: The Enthalpy of the Cr a) and C3 b) and the T_{max} of the Cr c) and C3 d) of raced and non-raced horses, and e) the DSC thermograms of the right Cr layer c from 3, 6 and 10 year old TB horses.

Enthalpy (J/mg)	Correlation with Age	Covariation with Age	Raced (Mean+/-SEM)	Non-raced (Mean+/-SEM)
Cr: Layer a				
Whole Peak	+0.15	NA	65.17+/-3.1	68.19+/-6.5
1st Peak	+0.13	NA	5.45+/-0.9	6.04+/-0.7
2nd Peak	+0.11	NA	59.72+/-3.4	62.15+/-6.1
Layer b				
Whole Peak	+0.41*	NS	59.72+/-4.2	74.42+/-8.1
1st Peak	-0.18	NA	3.21+/-0.4	4.02+/-0.5
2nd Peak	+0.43*	NS	56.51+/-3.9	69.36+/-7.4
Layer c				
Whole Peak	0.000	NA	69.49+/-3.3	67.98+/-4.1
1st Peak	-0.64**	Sig*	4.6+/-0.5	3.0+/-1.1
2nd Peak	+0.02	NA	64.85+/-3.5	64.94+/-4.2
Layer d				
Whole Peak	+0.33	NA	69.21+/-4.4	81.91+/-9.3
1st Peak	0.000	NA	3.77+/-0.4	3.57+/-0.3
2nd Peak	+0.33	NA	65.43+/-4.2	78.33+/-9.4
C3: Layer a				
Whole Peak	-0.26	NA	65.49+/-2.7	64.77+/-4.4
1st Peak	-0.15	NA	6.87+/-1.2	6.76+/-1.1
2nd Peak	-0.13	NA	58.62+/-3.3	58.01+/-4.2
Layer b				
Whole Peak	+0.21	NA	59.16+/-2.1	79.56+/-8.5*
1st Peak	-0.33	NA	2.65+/-0.4	4.21+/-1.01
2nd Peak	+0.17	NA	56.52+/-1.9	82.48+/-9.5*
Layer c				
Whole Peak	+0.04	NA	71.2+/-5.6	72.21+/-2.7
1st Peak	+0.72**	Sig**	3.97+/-0.4	4.01+/-1.4
2nd Peak	-0.12	NA	67.23+/-5.5	68.2+/-3.7
Layer d				
Whole Peak	+0.41*	NS	63.59+/-2.1	72.73+/-3.1*
1st Peak	-0.48*	Sig*	3.42+/-0.52	3.94+/-0.9
2nd Peak	+0.55*	Sig*	60.96+/-2.1	68.78+/-3.9

Table 3.20: Summary of the enthalpy of denaturation results in the Cr and C3 layers a-d of raced and non-raced horses. NS= Not significant. NA= Not applicable. Sig= Significant results= *($P<0.05$), **($P<0.01$).

T_{max} (°C)	Correlation with Age	Covariation with Age	Raced (Mean+/-SEM)	Non-raced (Mean+/-SEM)
Cr: Layer a				
Whole Peak	0.000	NA	59.88+/-0.7	59.48+/-1.3
1st Peak	+0.02	NA	58.27+/-0.8	59.28+/-1.4
2nd Peak	0.000	NA	99.72+/-1.3	97.54+/-1.2
Layer b				
Whole Peak	+0.02	NA	59.49+/-1.7	57.5+/-0.9
1st Peak	+0.02	NA	58.64+/-1.4	56.86+/-1.4
2nd Peak	-0.01	NA	91.66+/-1.6	87.5+/-0.85
Layer c				
Whole Peak	+0.28	NA	57.57+/-0.7	58.6+/-0.9
1st Peak	+0.24	NA	56.93+/-0.7	57.52+/-0.9
2nd Peak	0.000	NA	87.49+/-0.8	86.41+/-0.1
Layer d				
Whole Peak	-0.001	NA	57.47+/-0.8	59.17+/-0.9
1st Peak	-0.41*	NS	56.56+/-0.8	53.42+/-5.3
2nd Peak	-0.01	NA	87.77+/-1.6	85.23+/-0.7
C3: Layer a				
Whole Peak	+0.38	NA	59.06+/-0.9	60.12+/-1.1
1st Peak	+0.36	NA	58.84+/-0.6	58.7+/-0.8
2nd Peak	+0.52*	Sig*	94.37+/-2.1	95.85+/-3.2
Layer b				
Whole Peak	+0.06	NA	58.71+/-0.5	59.69+/-1.2
1st Peak	+0.045	NA	57.94+/-0.5	58.81+/-1.4
2nd Peak	+0.007	NA	87.34+/-0.7	87.52+/-1.2
Layer c				
Whole Peak	+0.41*	NS	57.14+/-0.6	58.9+/-0.8
1st Peak	+0.29	NA	55.95+/-0.8	57.58+/-0.6
2nd Peak	+0.08	NA	85.51+/-0.3	86.34+/-0.2
Layer d				
Whole Peak	+0.41	NA	58.2+/-1.2	58.37+/-1.3
1st Peak	+0.41*	NS	56.05+/-0.9	56.89+/-0.9
2nd Peak	-0.54*	Sig*	86.33+/-0.4	74.94+/-7.9

Table 3.21: Summary of the T_{max} results in the Cr and C3 layers a-d of raced and non-raced horses. NS= Not significant. NA= Not applicable.
Sig= Significant results= *($P<0.05$), **($P<0.01$).

3.1.4.4 Discussion

The Thermal Properties of Equine Bone Collagen

Two denaturation peaks are evident in the thermograms of equine bone collagen, which have been previously described in avian bone (Knott, Whitehead et al. 1995) and the MTLT (Knott, Tarlton et al. 1997). These are thought to represent two populations of collagen molecules, those that are relatively homogenous and less stable (1st peak), and those that are thermally more heterogeneous and more stable (2nd peak). These two populations are thought to differ in either the level of hydration and/or mineralisation (as previously mentioned) (Lees 1986) and are probably associated with the level and type of collagen cross-links within the collagen molecules (Flandin, Buffevant et al. 1984).

Relationships with Age

The relationships with age and the thermal properties of the collagen within this study are quite varied, especially compared to the relationships found to exist with age in sections 3.1.2 and 3.1.3. This may be in part due to the smaller number of equine samples, and hence a smaller age range (mean age 7 years), and limited statistical power in this part of the study compared to the other sections. Additionally, unlike the studies by Flandin *et al.*, (1984) and Knott *et al.*, (1997) there appeared to be no change in the shape of the peaks with age, which may be explained by the lack of very immature individuals included in this study, as such, less change may have occurred with age in these mature horses due to the reduction in the level of collagen turnover and modelling apparent at an young age.

Generally the T_{max} increased with age, suggesting that the triple helix is stabilising with age, which is further supported by the increase in mature collagen cross-links with age observed earlier in this study. Previous studies have been inconclusive on the effect of age on the enthalpy, with Flandin *et al.*, (1984) reporting no effect and Knott *et al.*, (1997) reporting an effect only in the mineralised tendon. Interestingly in this study, age was found to influence the enthalpy of denaturation. This effect like that noted in the study by Knott *et al.*, (1997), may not however be a true reflection of the ageing process

but instead reflect the effect of mineralisation on the collagen thermal properties. In support of this, within this study, the amount of calcium and inorganic phosphate was found to correlate positively with age (refer to Chapter Two).

The Effects of Exercise

High intensity exercise does appear to have an effect on the thermal properties of the collagen. Specifically, the enthalpy of denaturation of the whole and 2nd peak was significantly lower in layers b and d in the C3 of the raced compared to the non-raced horses. However, the T_{max} was not different in these samples which was unexpected, since previous studies have shown an altered T_{max} with an altered level of collagen cross-links (Bailey, Sims et al. 1993) and lysyl overhydroxylation (Notbohm, Mosler et al. 1992), which are similar to the cross-link modifications apparent in the racehorses in this study. The mechanism for this reduced enthalpy but lack of altered T_{max} is unknown, but it is speculated that a proportion of the collagen molecules are damaged and hence partially denatured, possibly due to the action of proteolytic enzymes such as MMP-2 and -9. Increased levels of MMP-2 and-9 have been shown within the racehorses in this study, supporting this suggestion. A similar finding was also demonstrated in degenerated equine tendon, with the enthalpy decreasing in relation to the extent of degeneration of the tendon with no change in T_{max} (Miles, Wardale et al. 1994).

3.1.5 General Discussion

Quantification of Equine Bone Collagen Turnover with Previously Unused Commercially Available Assays

The use of the commercially available assays to quantify PICP and Col2-3/4C$_{short}$ concentrations, markers of collagen synthesis and degradation respectively, in equine bone extract has not been previously documented. Part of this study was to investigate their potential as viable techniques to quantify these markers of collagen turnover. Both the PICP RIA (Orion Diagnostica, Finland) and the Collagen II 3/4C$_{short}$ ELISA (Ibex Diagnostics, USA) were found to cross-react with equine bone extract, hence providing evidence for their use in quantifying these markers in equine bone.

The Relationships Between Age and Bone Collagen Metabolism

Relationships with age and bone turnover in the midcarpal joint of TB horses are evident in this study and were found to be similar to that previously reported (Hank, Hoffmann et al. 1993; Price, Jackson et al. 1995; Brama, TeKoppele et al. 1998; Hiney, Potter et al. 2000; Aurich, Poole et al. 2002). In summary, levels of synthesis and degradation generally declined with age, reflecting a reduction in the level of modelling with maturation and growth. The relationships with age and the thermal properties of collagen were however, more varied and complex, with some positive and negative correlations with age being apparent, although in general the T_{max} increased with age suggesting a stabilisation of the triple helix with age. This further reflects a 'slowing down' of collagen turnover with age.

Clearly changes to the levels of collagen turnover alter with age, which must therefore be accounted for when biochemical markers are used to assess bone quality and aid diagnosis of skeletal diseases.

The Effect of Exercise of Bone Collagen Metabolism

The findings from the analysis of the collagenous matrix of raced and non-raced horses, suggest that high-intensity exercise is inducing an elevated collagen turnover, above the normal physiological response to exercise, which is resulting in the formation of a modified collagenous matrix.

Radial and Third Carpal Bone

Relatively few significant differences were seen between right and left bone values, suggesting that the mechanical load being applied to the midcarpal joint during high, medium to low intensity exercise is equally distributed. Any differences evident may be a chance finding or suggest that some horses preferentially lead with one forelimb more than the other as previously mentioned in Chapter Two.

There does appear to be a slightly greater level of collagen turnover in the C3 than the Cr, which may reflect the increased force absorbed by the C3 during carpal flexion (Barr 1994; Back, Schamhardt et al. 1995) and may give some explanation to the more frequently documented failure of the C3 in racing thoroughbreds (O'Brien 1977; O'Brien, DeHaan et al. 1985; De Haan, O'Brien et al. 1987; Young, O'Brein et al. 1988).

Cortical and Trabecular Regions of the Bone

Racing and race-training appears to be inducing increased levels of collagen turnover within both the cortical and trabecular regions of the Cr and C3. Interestingly however, the greatest significant differences between the two groups of horses was evident in the deeper, trabecular regions of the bone, which presumably reflects the higher turnover rate and hence the more immature state of the trabecular bone in these racehorses. Previous studies on the subchondral trabecular bone from the femoral neck of OA and OP patients, have shown increased MMP-2 and ALP levels compared to the trabecular bone from normal specimens (Mansell, Tarlton et al. 1997; Mansell and Bailey 2003) and within the OP patients, increased collagen cross-links, PICP and hydroxylysine concentrations were also documented, demonstrating a higher rate of matrix turnover in diseased trabecular bone which may possibly increase bone fragility (Knott, Whitehead et al. 1995).

Results similar to that reported in the OA and OP subchondral trabecular bone have been demonstrated here within the racehorse Cr and C3. Failure of the subchondral bone commonly observed in the Cr and C3 of racehorses may therefore result from its lack of support by a less resilient trabecular bone.

Summary

In summary, the results of this study demonstrate an elevated bone remodelling process in the Cr and C3 of horses that are subject to high-intensity exercise, which is resulting in a modified collagenous matrix. The increased PICP, BAP and immature cross-link content indicate that 'new' bone collagen is being synthesised in the racehorses. However, these collagen fibrils are unable to form mature covalent cross-links due to the increased activity of the degrading proteolytic enzymes, including MMP-2 and –9. This may result in bone that has less tensile strength, which is supported by the increased lysine hydroxylation and as such result in a bone that is biomechanically weaker and hence more susceptible to failure.

3.2 EXPERIMENTAL INVESTIGATIONS OF BONE COLLAGEN THERMAL PROPERTIES

As previously mentioned, to date, limited information exists regarding the thermal characteristics of the collagen in cortical and trabecular bone. DSC can be used to quantify the thermal properties of bone collagen, however, calcified bone has a very high denaturation temperature, approximately, 155°C (Kronick and Cooke 1996) and as such calcified bone could not to be analysed due to the absence of a measurable denaturation endotherm within the temperature ranges available. Hence the tissue samples had to be decalcified prior to DSC analyses and the best process for this to yield reproducible data had to be determined.

In addition, an interesting feature of the equine bone collagen thermograms is the appearance of two denaturation peaks. However, it has not been ascertained in equine bone collagen whether these are actually multiple denaturation events hence validation of this was required. Another interesting feature of the thermograms, which only became apparent during the analysis of the equine bone samples outlined in section 3.1.4, was an apparent decrease in the T_{max} of these specimens compared to the specimens which had been analysed in the following Investigation A (section 3.2.1). This was thought to be due to the effect of salt solutions, i.e. the EDTA/$CaCl_2$ used during the decalcification procedure, altering the thermal properties of the collagen. It is known that the pH of a solution can determine the state of a protein (Privalov 1979), mainly by affecting the electrostatic and hydrophobic interactions in the protein molecules (Melander and Horvath 1977). Although previous studies have examined the effect of salts on proteins, including collagen (Lim 1976; Burjanadze and Bezhitadze 1992; Komsa-Penkova, Koynova et al. 1996; Kaushik and Bhat 1999), most of these have been conducted on non-calcifying collagen in solution and to date the effect of salts on non-soluble calcifying collagenous tissues has received scant attention.

In order to understand the behaviour of decalcified bone collagen during thermal denaturation, experiments were performed:

A) Decalcification procedure of equine bone

Aims: To determine the influence of the decalcification procedure and the different methods to remove residual EDTA, on the denaturation events of type I equine bone collagen.

B) The nature of the denaturation peaks of equine type I bone collagen

Aim: To determine if the thermogram consists of a single or multiple denaturation event.

C) The effect of salt solutions on the thermal characteristics of type I collagen

Aim: To determine the effect of salt solutions on the thermal characteristics of calcified and non-calcified type I collagenous tissues.

3.2.1 A) An Investigation into the Decalcification Procedure of Equine Bone

3.2.1.1 Materials and Methods

a) Decalcification with EDTA and removal of residual EDTA:

Pulverised equine cortical bone (layer a) was used in the experiments and subjected to the following decalcification procedures:

Exp I: Aliquots of pulverised bone were decalcified for 4, 8, 24, 28, 32 and 48hrs respectively in 0.5M EDTA (tetrasodium salt) pH 7.5 (at a concentration of 10mg (ww)/5ml EDTA) and subsequently washed in deionised water (dH_2O) (x2) to remove the residual EDTA.

Exp II: Aliquots of pulverised bone were decalcified for 4, 8, 24, 28, and 32hrs respectively in 0.5M EDTA (tetrasodium salt) pH 7.5 (10mg (ww)/5ml EDTA). These aliquots of bone were subsequently dialysed against 0.02M acetic acid (HAc) for the same time period they had been decalcified with EDTA i.e. 4, 8, 12, 24, 28 and 32hrs respectively, to remove the residual EDTA.

Exp III: Aliquots of pulverised bone were decalcified in 0.5M EDTA (tetrasodium salt) pH 7.5 (10mg (ww)/5ml EDTA) for 2 days with a change of EDTA solution after the 1st day. The samples were then washed in 10ml 2M $CaCl_2$ (pH 4.4) for 1hr

to remove the EDTA and subsequently washed with dH_2O (x2) to remove the residual $CaCl_2$.

Thermal Analysis - Differential Scanning Calorimetry:

Thermal analysis was performed on the decalcified bone aliquots using DSC as previously described (refer to section 3.1.4.2.3). Each experiment was performed in duplicate.

Calcium and Inorganic Phosphate Quantification

The total Ca and Pi content of the decalcified tissues was obtained to determine any residual mineral. The method used was one previously described in Chapter Two section 2.2.3.

3.2.1.2 Results

Exp I: During early analysis of the demineralised bone washed only in dH_2O x2, a sharp exotherm at the end of DSC thermogram was evident (peaking at approximately 120°C), as shown in Figure 3.33. This is uncharacteristic of collagen thermograms and made quantification of the 2nd peak difficult. This uncharacteristic exotherm was thought to be due to the residue of EDTA in the sample. Testing a small aliquot of EDTA on the DSC created the same exotherm further supporting this theory. Demineralising bone in EDTA and washing with dH_2O x2 clearly did not remove all the residual EDTA.

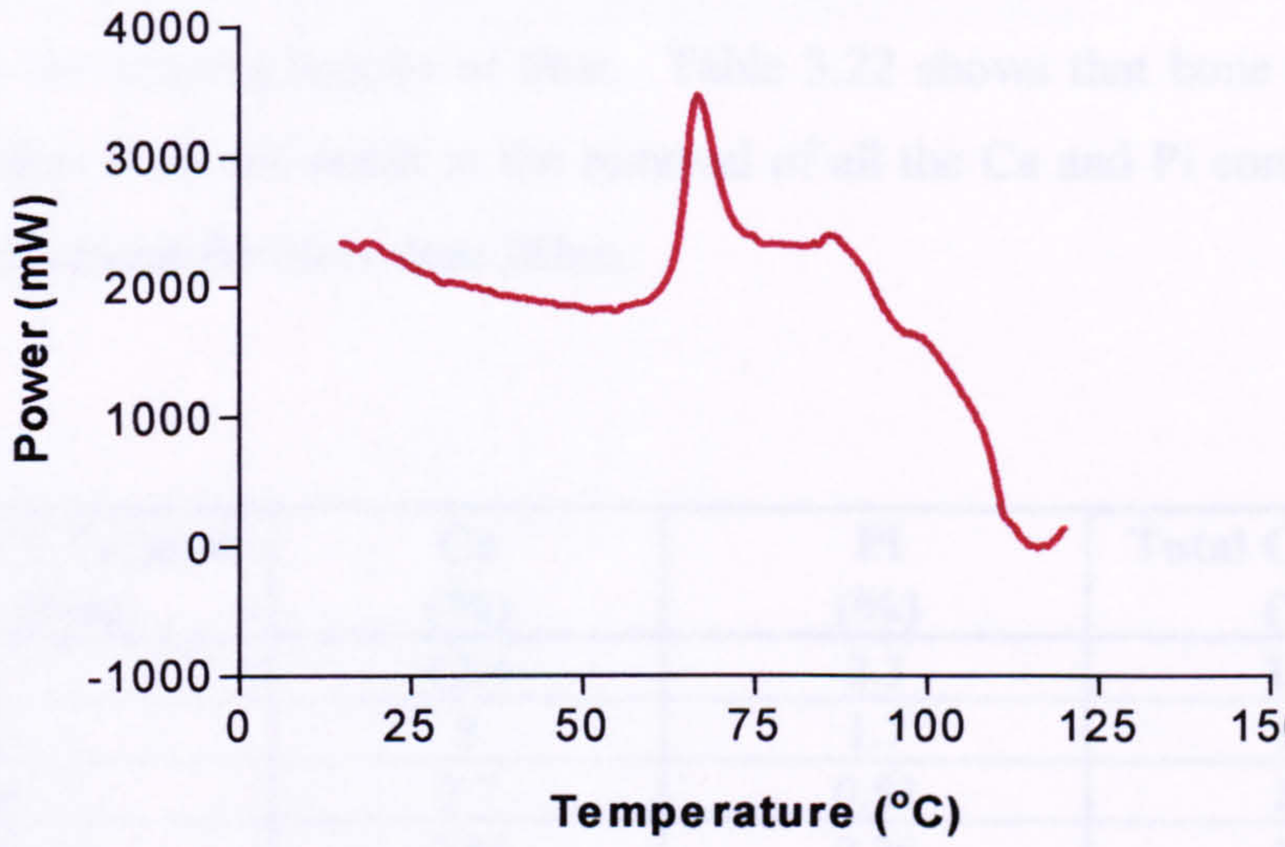


Figure 3.33: A typical DSC thermogram of EDTA treated equine bone, demonstrating an exotherm peaking at approximately 120°C.

Exp II: After EDTA treatment bone aliquots were subsequently dialysed against 0.02M acetic acid (HAc) to ensure the removal of any residual EDTA. As a consequence of this, DSC thermograms did not exhibit the exotherm shown in Figure 3.36 and the original investigation to determine the length of time needed to demineralise bone to yield re-producible results could be undertaken.

Figure 3.34 illustrates the thermograms for bone demineralised in EDTA and subsequently dialysed against HAc at varying times. The T_{max} of peak 1 in the 4hr and 24hr bone collagen are variable, with T_{max} being higher in the 4hr comparable to the 24hr bone. However, the T_{max} of the 28hr and 32hr bone only have a marginal difference of 0.1°C. This is also reflected in Figure 3.35a, where the line of best fit begins to plateau between 24 and 28hrs. Additionally, Figure 3.35b illustrates the enthalpy of the bone at the varying periods of demineralisation. The enthalpy for the 4, 8 and 12hr demineralised bone is low, comparable to that of the 24hr and 28hr bone, suggesting that only the less stable collagen molecules have been denaturated in the 4, 8 and 12hr bone at this temperature (0-130°C).

The total Ca and Pi was quantified to assess the effectiveness of demineralisation of bone with EDTA for varying lengths of time. Table 3.22 shows that bone demineralised in EDTA for 8hrs does not result in the removal of all the Ca and Pi comparable to bone that is demineralised for more than 28hrs.

EDTA Treated (hrs)	Ca (%)	Pi (%)	Total Ca and Pi (%)
8hrs:	12.4	2.3	**14.8**
12hrs:	8	1.7	**9.7**
28hrs:	2.7	0.82	**3.5**
48hrs:	0.41	0.26	**0.7**

Table 3.22: The Ca and Pi content of equine bone following different extends of demineralisation by washing in EDTA.

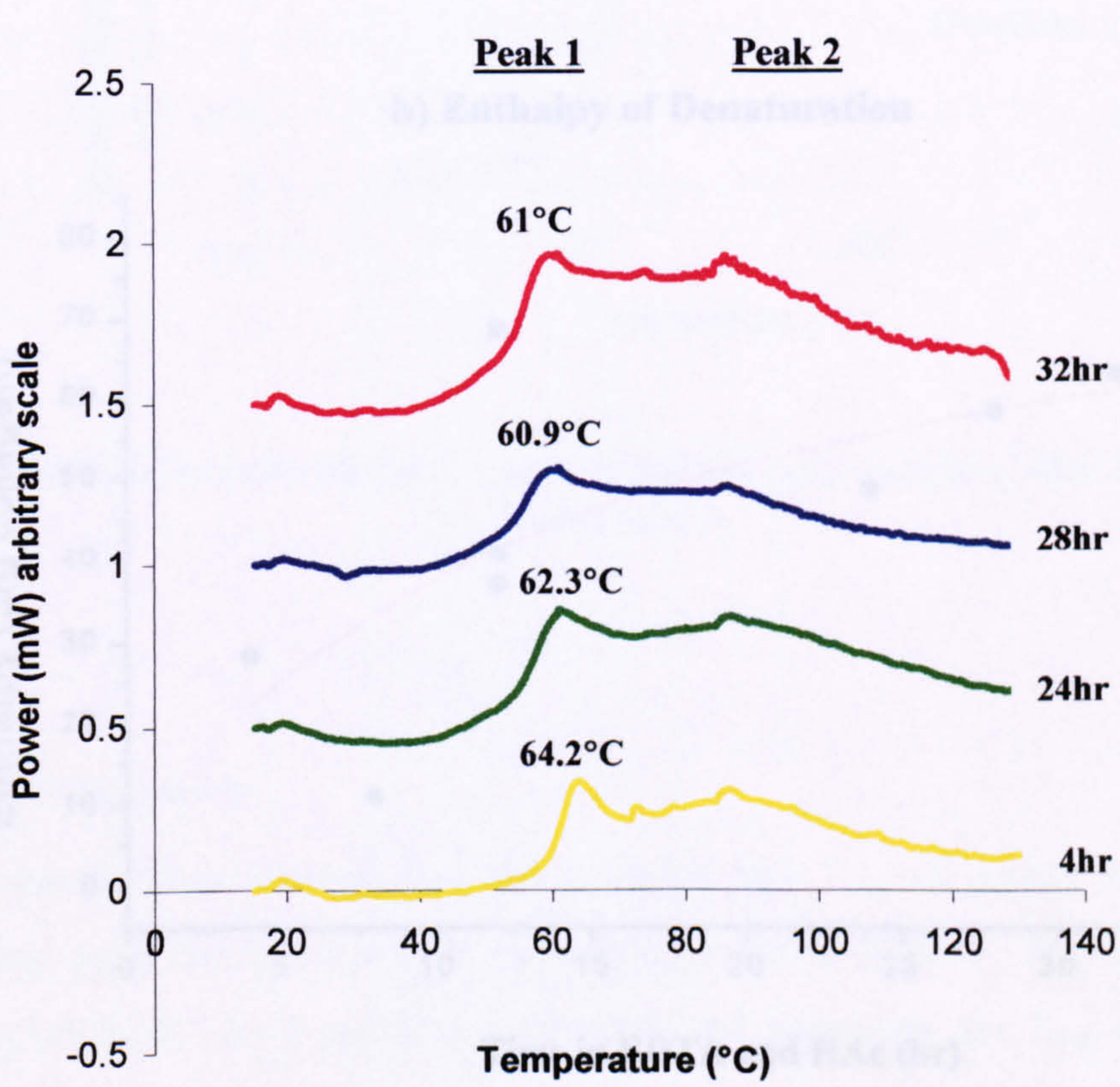
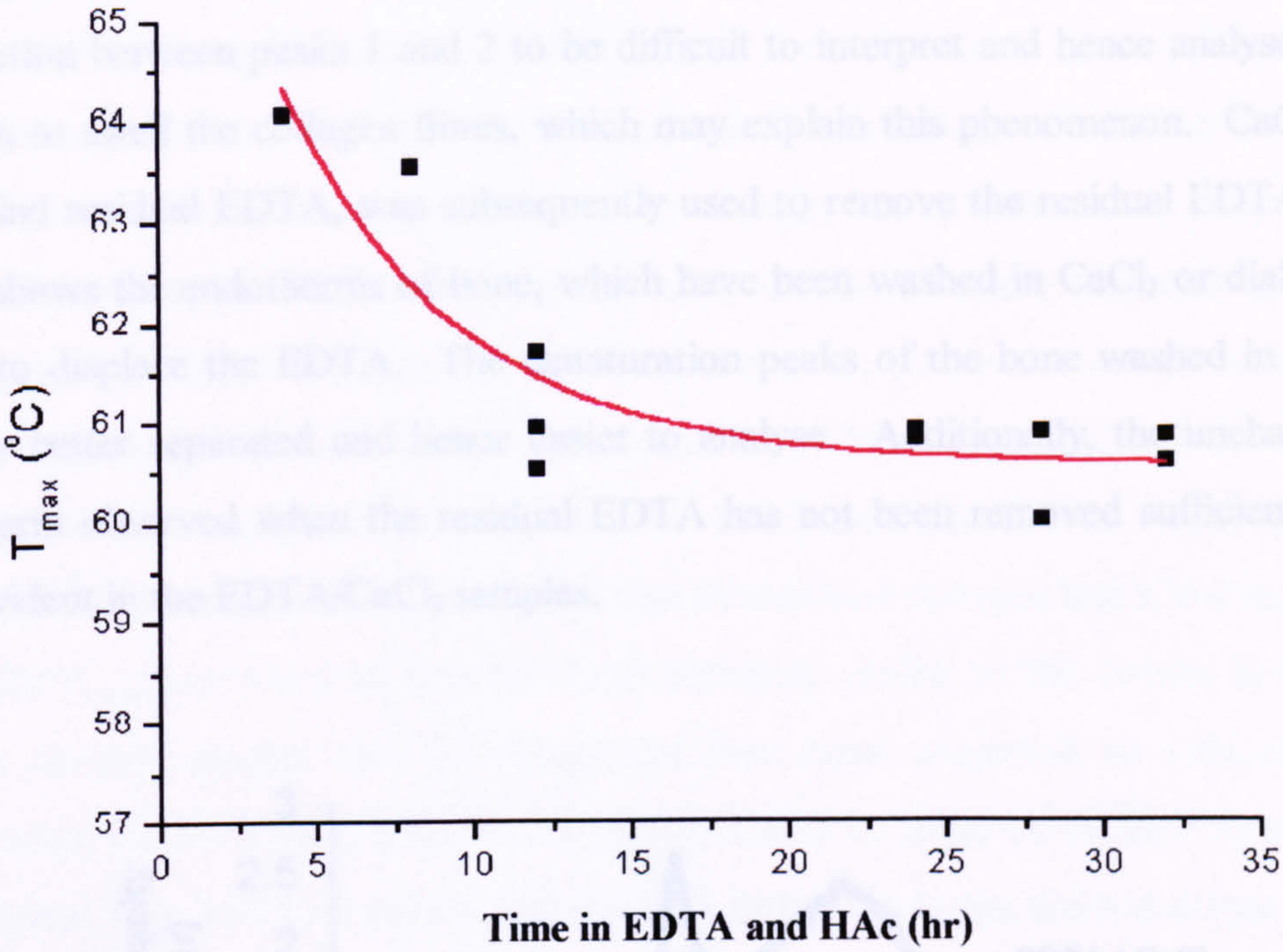


Figure 3.34: Effect of various EDTA and HAc treatment times on the T_{max} of equine bone collagen.

a) Temperature of Denaturation



b) Enthalpy of Denaturation

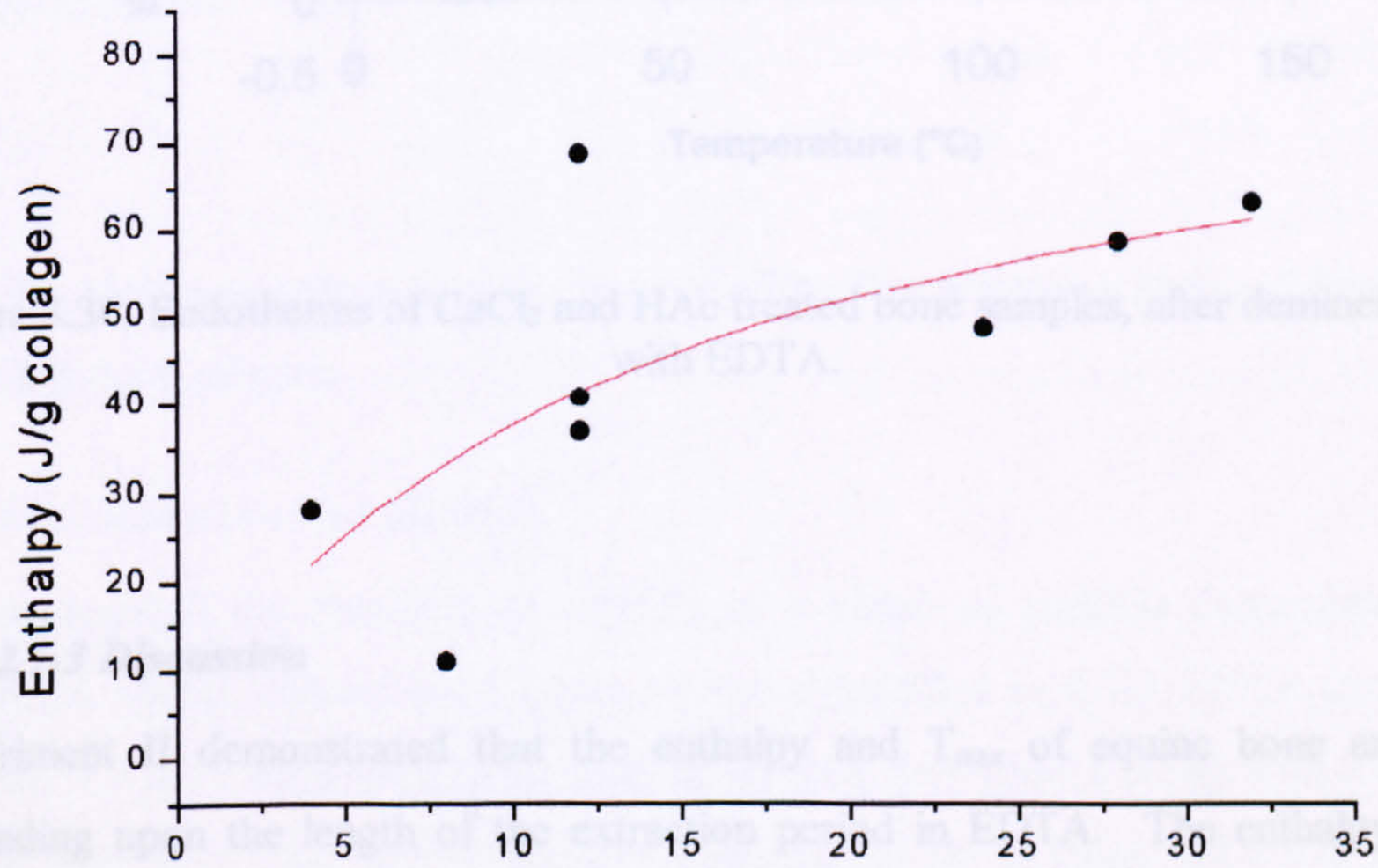


Figure 3.35: Effect of various EDTA and HAc treatment times on a) T_{max} and b) the enthalpy of denaturation.

Exp III: One characteristic of the endotherms of the bone treated with EDTA and subsequently dialysed with HAc was the broadness of the peaks, often causing the distinction between peaks 1 and 2 to be difficult to interpret and hence analyse. HAc is known to swell the collagen fibres, which may explain this phenomenon. $CaCl_2$, which will bind residual EDTA, was subsequently used to remove the residual EDTA. Figure 3.36 shows the endotherms of bone, which have been washed in $CaCl_2$ or dialysed with HAc to displace the EDTA. The denaturation peaks of the bone washed in $CaCl_2$ are clearly better separated and hence easier to analyse. Additionally, the uncharacteristic exotherm observed when the residual EDTA has not been removed sufficiently is also not evident in the EDTA/$CaCl_2$ samples.

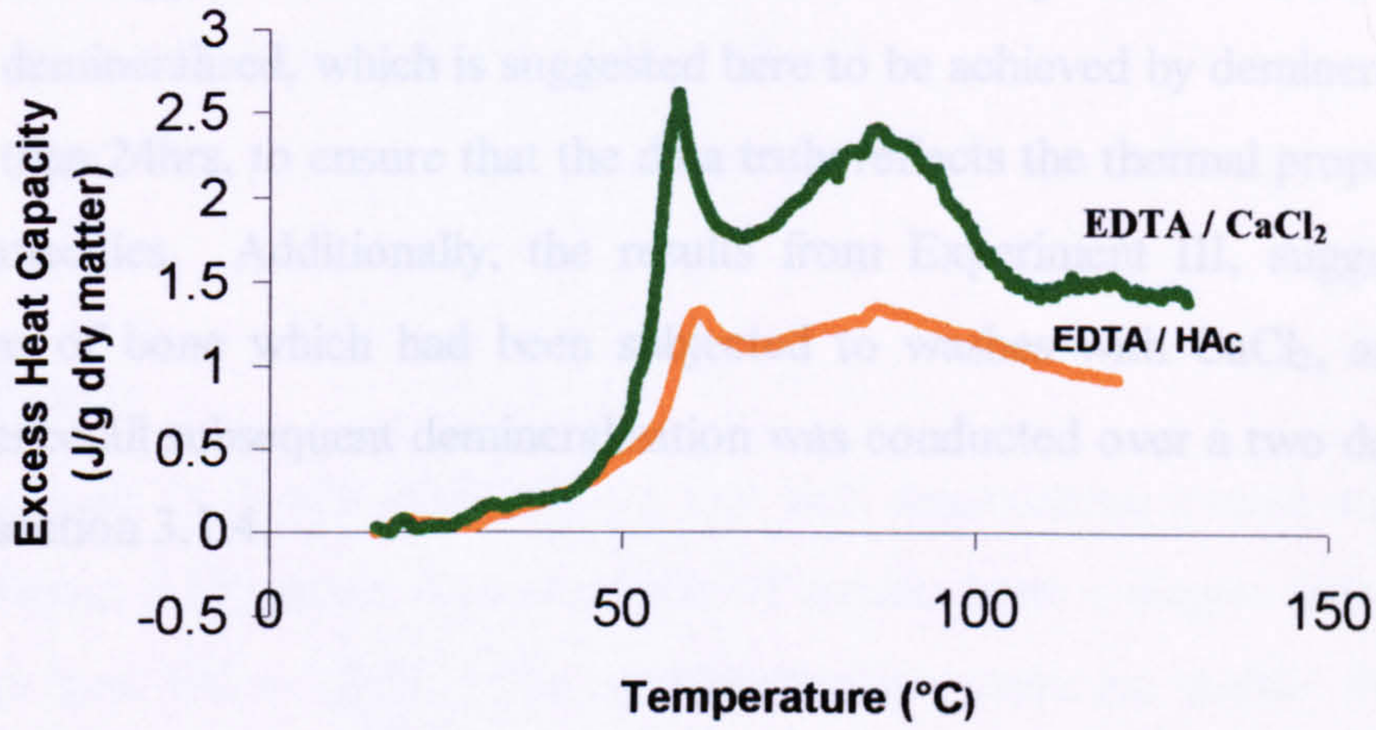


Figure 3.36: Endotherms of $CaCl_2$ and HAc treated bone samples, after demineralisation with EDTA.

3.2.1.3 Discussion

Experiment II demonstrated that the enthalpy and T_{max} of equine bone are altered depending upon the length of the extraction period in EDTA. The enthalpy of bone extracted in EDTA for 4 and 8hrs is very low and conversely the T_{max} is high compared to the enthalpy and T_{max} of bone extracted for greater than 24hrs. This together with the total calcium and inorganic phosphate results of bone treated for these periods in EDTA, indicate that the demineralisation of bone in EDTA for 4 or 8hrs is not sufficient to

remove all the mineral. Evidence exists to suggest that mineralisation increases the thermal stability of the collagen molecules (Bonar and Glimcher 1970; Kronick and Cooke 1996; Knott, Tarlton et al. 1997). It was found by Bonar *et al.*, (1970) that bone could be heated to 100°C, cooled for 24hrs and demineralised with EDTA for four days with no change in either the wide or low angle X-ray diffraction pattern i.e. the molecular structure. Conversely, heating after demineralisation, followed by the same annealing and EDTA treatments, caused the low-angle pattern to vanish, with retention of the high angle pattern (Bonar and Glimcher 1970). Specifically, DSC studies on bovine bone collagen (Kronick and Cooke 1996) and mineralised turkey leg tendon (Knott, Tarlton et al. 1997), have found that mineralised collagen had a low enthalpy and a higher T_{max} compared to demineralised collagen, similar to the results in this study. These studies suggest that it is essential that bone subjected to DSC analysis is completely demineralised, which is suggested here to be achieved by demineralising bone for greater than 24hrs, to ensure that the data truly reflects the thermal properties of the collagen molecules. Additionally, the results from Experiment III, suggest that the thermograms of bone which had been subjected to washes with $CaCl_2$, are easier to interpret, hence all subsequent demineralisation was conducted over a two day period as outlined in section 3.1.4.

3.2.2 B) An Investigation into the Nature of the Denaturation Peaks of Equine Type I Bone Collagen

3.2.2.1 Materials and Methods

To determine if the thermogram consists of a single or multiple denaturation event, pulverised equine cortical bone (layer a) was decalcified in 0.5M EDTA (tetrasodium salt) pH 7.5 for 2 days (at a concentration of 10mg (ww)/5ml EDTA) with a change of EDTA after the 1st day, and subsequently washed for 1hr in 10mg 2M $CaCl_2$ to remove any residual EDTA and then washed thoroughly with dH_2O (x2). Thermal analysis was subsequently performed using DSC as previously described (refer to section 3.1.4.2.3), with the following modifications for each experiment. Each experiment was performed in duplicate.

a) A standard run from 5°C to 130°C of an aliquot of decalcified bone was undertaken (1st run). The sample was cooled and then re-heated to 130°C (2nd run).

b) An aliquot of decalcified bone was partially heated from 5°C to 57°C (the centre of the main denaturation peak (1st peak) as ascertained from previous runs) and cooled (1st run), re-heated to 130°C, cooled (2nd run) and subsequently re-heated to 130°C (3rd run).

c) An aliquot of decalcified bone was partially heated from 5°C to 70°C (the end of the 1st peak as ascertained from previous runs) and cooled (1st run), re-heated to 130°C, cooled (2nd run) and subsequently re-heated to 130°C (3rd run).

d) An aliquot of decalcified bone was partially heated from 5°C to 80°C (10°C after the end of the 1st peak) and cooled (1st run), re-heated to 130°C, cooled (2nd run) and subsequently re-heated to 130°C (3rd run).

3.2.2.2 Results

The thermograms in Figure 3.37 indicate that two denaturation events are occurring. Part a of Figure 3.37 shows a thermogram of equine bone collagen heated during a standard run from 5°C to 130°C. The two denaturation peaks are visible. Part b shows that when the same sample is heated to 57°C (1st run), cooled and reheated (2nd run), the collagen that was not denatured during the 1st run is subsequently denatured when reheated. The peaks are not visible in the 3rd run, demonstrating that all the collagen molecules have been denatured.

Parts c and d of Figure 3.37 show that partial heating to the various temperatures, 70°C and 80°C respectively (1st run), results in a peak in the thermogram at approximately 60°C (1st peak) which when this same sample is reheated to 130°C (2nd run) is not observed on the thermograms, demonstrating that some of the collagen molecules are fully denaturated at this temperature. During the 2nd run a second peak is evident at approximately 85°C, which again when reheated to 130°C is not observed (3rd run). This demonstrates that a second denaturation of collagen molecules is occurring. The two denaturation events seen in the thermograms at 60°C and 85°C are therefore distinct.

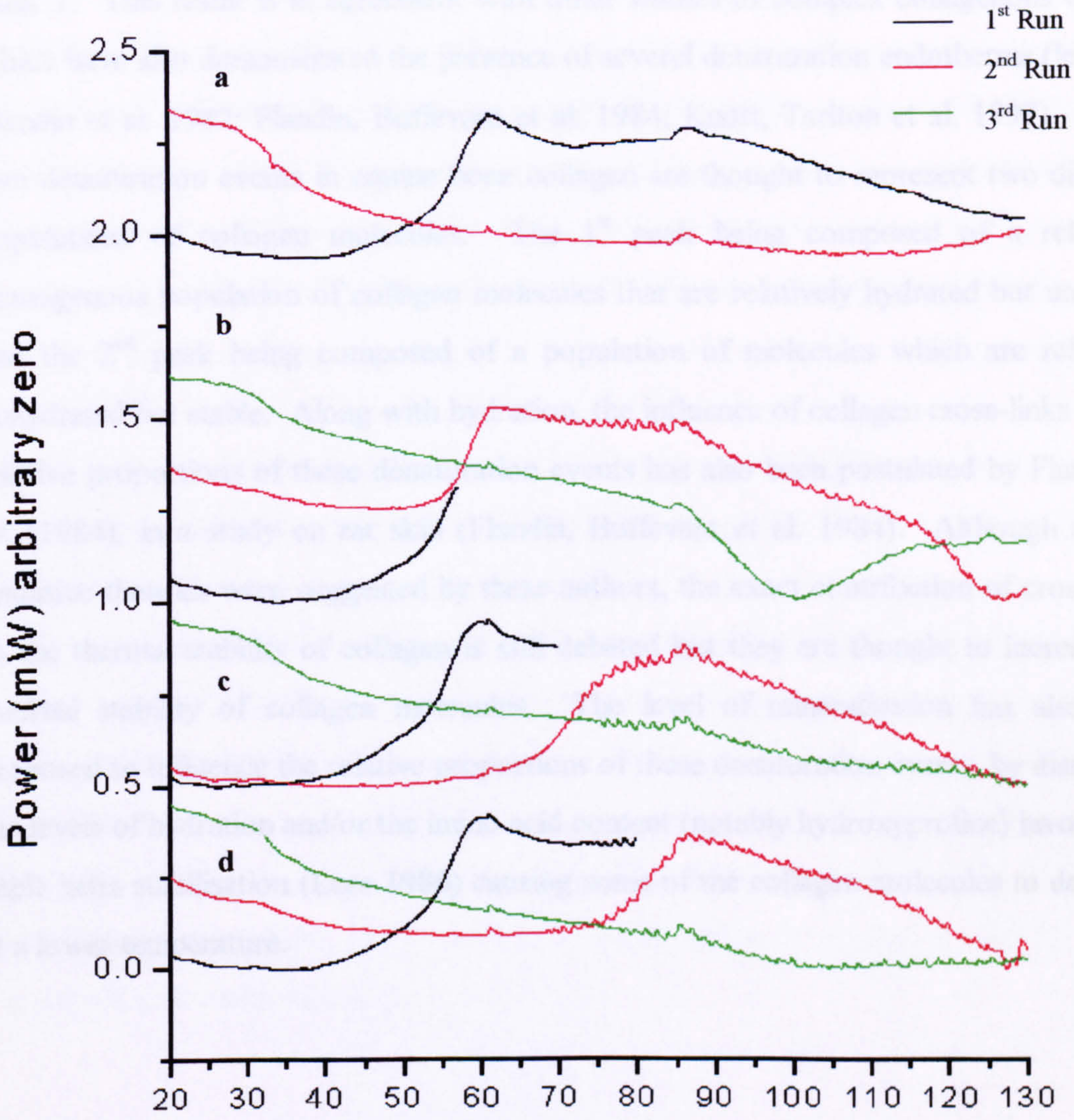


Figure 3.37: The effect of partial heating on equine bone collagen, demonstrating that denaturation consists of more than one event.

3.2.2.3 Discussion

The results from this investigation demonstrate that the broader section of the thermogram (peak 2) was a separate denaturation event to the main, sharper endothermic peak 1. This result is in agreement with other studies of complex collagenous tissues, which have also demonstrated the presence of several denaturation endotherms (le Lous, Flandin et al. 1982; Flandin, Buffevant et al. 1984; Knott, Tarlton et al. 1997). These two denaturation events in equine bone collagen are thought to represent two different populations of collagen molecules. The 1st peak being composed of a relatively homogenous population of collagen molecules that are relatively hydrated but unstable, and the 2nd peak being composed of a population of molecules which are relatively dehydrated but stable. Along with hydration, the influence of collagen cross-links on the relative proportions of these denaturation events has also been postulated by Flandin *et al*, (1984), in a study on rat skin (Flandin, Buffevant et al. 1984). Although several tentative theories were suggested by these authors, the exact contribution of cross-links to the thermal stability of collagen is still debated but they are thought to increase the thermal stability of collagen molecules. The level of mineralisation has also been proposed to influence the relative proportions of these denaturation events, by disrupting the levels of hydration and/or the imino acid content (notably hydroxyproline) involved in triple helix stabilisation (Lees 1986) causing some of the collagen molecules to denature at a lower temperature.

3.2.3 C) An Investigation into the Effect of Salt Solutions on the Thermal Characteristics of Type I Collagen

3.2.3.1 Materials and Methods

To determine the effect of salt solutions on the thermal characteristics of calcified and non-calcified type I collagenous tissues, rat tail tendon, a non-calcified tissue composed principally of type I collagen fibres, the proximal region of turkey leg tendon and the cortical region (layer a) of equine bone, both calcified type I collagenous tissues were used in the experiments. Pulverised aliquots of rat tail tendon, turkey leg tendon and equine bone, were subjected to the following treatments;

Exp 1: 0.5M EDTA (tetrasodium salt) pH 7.5 for 2 days (10mg (ww)/5ml EDTA) with a change of EDTA after the 1st day, and subsequently washed in dH_2O to remove the EDTA.

Exp 2: 0.5M EDTA (tetrasodium salt) pH 7.5 for 2 days (10mg (ww)/5ml EDTA) with a change of EDTA after the 1st day, and subsequently washed for 1hr in 10ml 2M $CaCl_2$ (pH 4.4) to remove any residual EDTA and then washed with dH_2O (x2).

Exp 3: 0.5M EDTA (tetrasodium salt) pH 7.5 for 2 days (10mg (ww)/5ml EDTA) with a change of EDTA after the 1st day, and subsequently washed in dH_2O (x2) to remove the EDTA. 10µl of 2M $CaCl_2$ (pH 4.4) was added to the DSC aluminium pans.

Rat tail tendon only was subjected to the following treatments. These experiments could not be undertaken on the turkey leg tendon or equine bone as there was no decalcification step;

Exp 4: Washed for 1hr in 2M $CaCl_2$ and then washed thoroughly with dH_2O.

Exp 5: 10µl of 2M $CaCl_2$ was added to the DSC aluminium pans.

Exp 6: No treatment.

Thermal analysis was performed on the rat tail tendon, turkey leg tendon and equine bone aliquots using DSC as previously described (refer to section 3.1.4.2.3). Each experiment was performed in duplicate.

3.2.3.2 Results

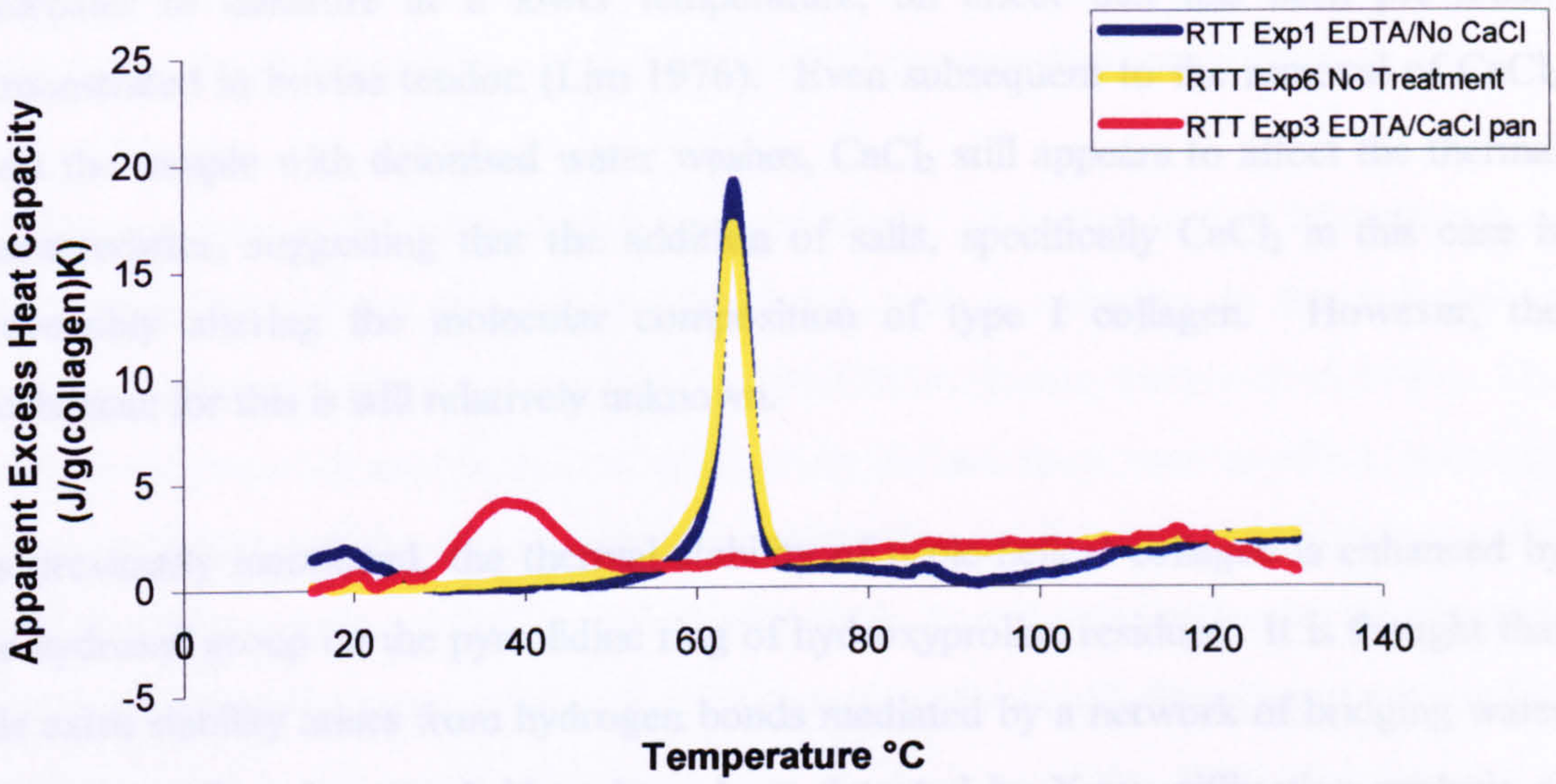
Table 3.23 shows the thermal characteristics of rat tail tendon, turkey leg tendon and equine bone subjected to experiments 1 to 6 and the previously published thermal characteristics of these tissues as a comparison. Washing with $CaCl_2$ (and subsequently with dH_2O) or adding $CaCl_2$ to the aluminium pan, did appear to affect the thermal characteristics of all the type I collagenous tissues analysed, particularly the T_{max}, which was reduced in the presence of $CaCl_2$. This is further illustrated in Figure 3.38 a and b. Additionally, in turkey tendon and to a lesser extent in equine bone, $CaCl_2$ appeared to alter the enthalpy of denaturation between the two peaks, with the enthalpy of peak 1

decreasing with a concomitant increase in the enthalpy of the second peak. EDTA treatment alone, however, does not appear to have a fundamental effect on the thermal characteristics of the tissues.

Sample	Enthalpy (J/g collagen)			T_{max} (°C)
	Whole Peak	1st Peak	2nd Peak	1st Peak
Rat Tail Tendon				
Published Thermal Characteristics (Miles et al., 1999):	70	-	-	60-65
Exp 1 (EDTA/wash dH_2O):	75.9	-	-	64.3
Exp 2 (EDTA/wash $CaCl_2$ & dH_2O):	72.8	-	-	41.9
Exp 3 (EDTA/wash dH_2O/$CaCl_2$ in pan):	34.3	-	-	37.6
Exp 4 (No EDTA/ wash $CaCl_2$ & dH_2O):	21.6	-	-	58.2
Exp 5 (No EDTA/ $CaCl_2$ in pan):	40.5	-	-	45.8
Exp 6 (No treatment):	79.5	-	-	64.2
Turkey Tendon				
Published Thermal Characteristics of Proximal Turkey Tendon (decalcified) (Knott et al., 1997):	68.4	53.8	10.4	67.5
Exp 1 (EDTA/wash dH_2O):	60.4	26.7	33.7	71.2
Exp 2 (EDTA/wash $CaCl_2$ & dH_2O):	74.4	20.6	53.8	60.5
Exp 3 (EDTA/wash dH_2O/$CaCl_2$ in pan):	86.3	11.1	96.8	51.9
Equine Bone				
Published Thermal Characteristics of Avian Bone (decalcified) (Knott et al., 1995):	41.1	14.7	26.4	69.3
Exp 1 (EDTA/wash dH_2O):	78.1	9.5	68.5	65.1
Exp 2 (EDTA/wash $CaCl_2$ & dH_2O):	65.7	6.36	59.4	59.5
Exp 3 (EDTA/wash dH_2O/$CaCl_2$ in pan):	64.2	5.6	58.6	52

Table 3.23: The thermal characteristics of calcifying and non-calcifying type I collagenous tissues subjected to various treatments with EDTA and $CaCl_2$.

a) Rat Tail Tendon



b) Turkey Tendon and Equine Bone

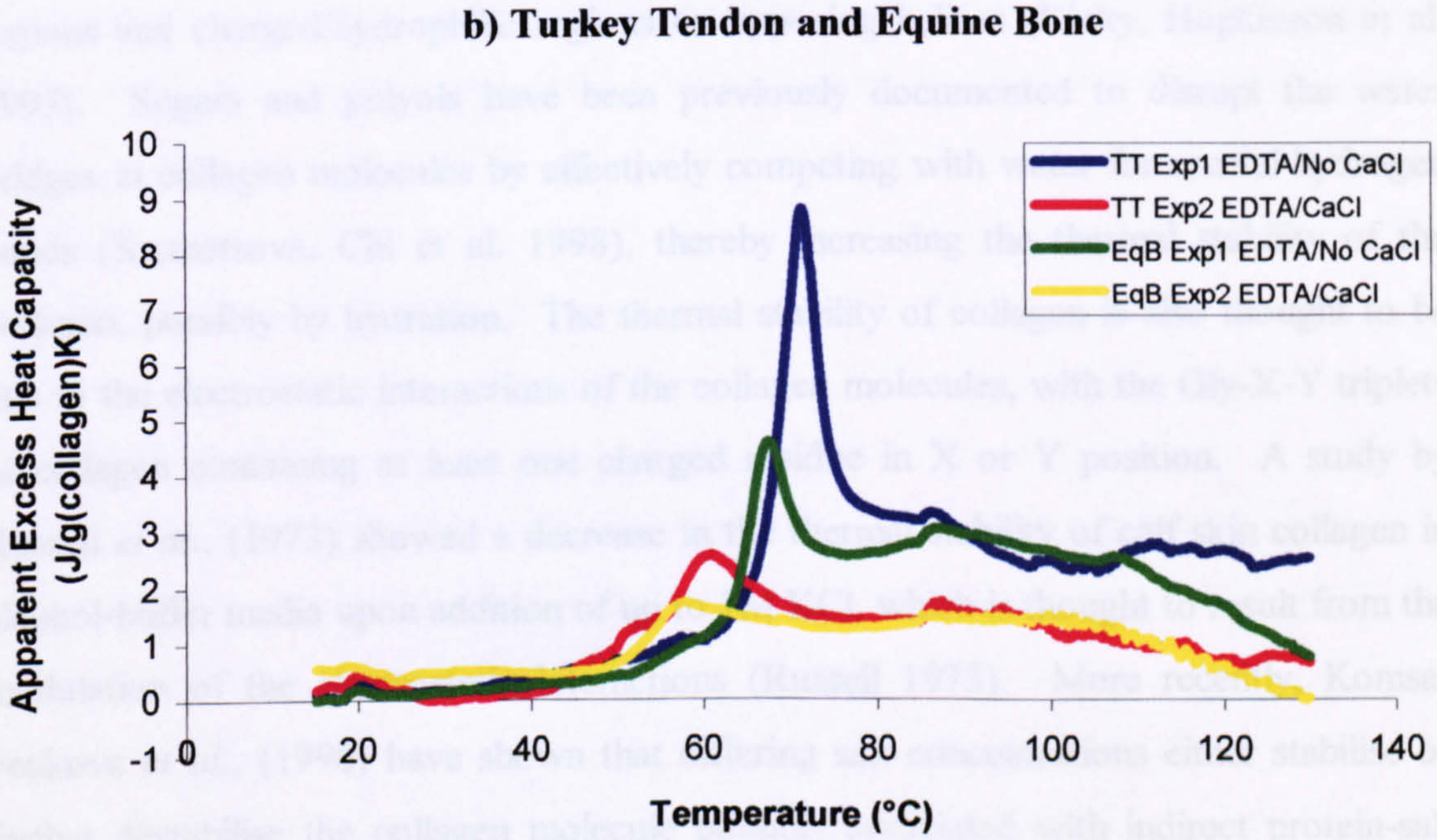


Figure 3.38: Endotherms of a) rat tail tendon (RTT) and b) turkey tendon (TT) and equine bone (EqB) subjected to various treatments with EDTA and $CaCl_2$.

3.2.3.3 Discussion

$CaCl_2$ appears to have a de-stabilising effect on calcifying collagen, allowing the collagen molecules to denature at a lower temperature, an effect that has been previously demonstrated in bovine tendon (Lim 1976). Even subsequent to the removal of $CaCl_2$ from the sample with deionised water washes, $CaCl_2$ still appears to affect the thermal characteristics, suggesting that the addition of salts, specifically $CaCl_2$ in this case is irreversibly altering the molecular composition of type I collagen. However, the mechanism for this is still relatively unknown.

As previously mentioned, the thermal stability of triple-helical collagen is enhanced by the hydroxyl group on the pyrrolidine ring of hydroxyproline residues. It is thought that this extra stability arises from hydrogen bonds mediated by a network of bridging water molecules and such water bridges have been detected by X-ray diffraction analysis of crystalline collagen (Bella, Eaton et al. 1994). They consist typically of two water molecules that link a hydroxyproline side-chain of one strand to a main-chain carbonyl of another strand of the triple helix, typically by forming alignment of both hydrophobic regions and charged/hydrophilic regions on apposing helices (Kielty, Hopkinson et al. 1993). Sugars and polyols have been previously documented to disrupt the water bridges in collagen molecules by effectively competing with water for crucial hydrogen bonds (Kuznetsova, Chi et al. 1998), thereby increasing the thermal stability of the collagen, possibly by hydration. The thermal stability of collagen is also thought to be due to the electrostatic interactions of the collagen molecules, with the Gly-X-Y triplets of collagen containing at least one charged residue in X or Y position. A study by Russell *et al.*, (1973) showed a decrease in the thermal stability of calf skin collagen in alcohol-buffer media upon addition of up to 1M KCl, which is thought to result from the modulation of the electrostatic interactions (Russell 1973). More recently, Komsa-Penkova *et al.*, (1996) have shown that differing salt concentrations either stabilise or further destabilise the collagen molecule possibly associated with indirect protein-salt interactions exerted via competition for water molecules between ions and the protein surface (generally termed the Hoffmeister effect) (Komsa-Penkova, Koynova et al. 1996). Although the results from this study are unable to contribute to the mechanisms involved in the de-stabilising effect of salts on collagen, (and a satisfactory explanation of

the data requires a thorough investigation beyond the scope of this thesis), the results do provide further evidence that during the de-calcification procedure of equine bone, $CaCl_2$ may be altering the hydrogen bonding and/or the electrostatic interactions of demineralised collagenous tissues, thus altering the denaturation temperature.

Previous studies on the distal region (non-calcifying) of turkey tendon have shown an effect of EDTA on the thermal characteristics, with a reduction in the enthalpy of denaturation being evident in those treated with EDTA (Knott, Tarlton et al. 1997). The similar experiment on rat tail tendon in this study did not show such an effect, however, the study by Knott *et al.*, (1997) only showed an effect on the enthalpy of peaks 1 and 2 and not on the whole peak (i.e. peak 1 plus peak 2) and since only one denaturation event occurs in rat tail tendon it is difficult to compare the results from these two studies.

The results from this study may have implications for the decalcification procedure utilised on bone, which is to be subjected to thermal analysis via DSC. Although this may therefore question the reliability of the results represented in section 3.1.4. all specimens were subjected to the same decalcification procedure, and hence this effect of $CaCl_2$ was thought not to influence the results represented in that section.

CHAPTER FOUR

The Biomechanics and Collagen Metabolism of the Dorsolateral and Dorsomedial Branches of the Medial Palmar Intercarpal Ligament

4.1 INTRODUCTION

The occurrence of tearing of the medial palmar intercarpal ligament (MPICL) was first documented in 1990 by Kannegieter *et al.*, and McIlwraith (Kannegieter and Burbidge 1990; McIlwraith 1990) and its association with carpal joint disease has since been postulated by various authors (McIlwraith 1992; Kannegieter and Colgan 1993; Phillips and Wright 1994; Whitton, Kannegieter et al. 1999). However, the pathogenesis of MPICL tearing is still unknown. It has been proposed that MPICL tearing causes instability in the midcarpal joint and hence contributes to osteochondral damage, particularly within the Cr and C3 (Kannegieter and Colgan 1993). Previous studies have demonstrated a relationship between remodelling of the dorsal aspect of the Cr and tearing of the MPICL (Phillips and Wright 1994). It has also been shown that clinical signs of carpal joint disease were more severe in joints with tearing than in joints with similar osteochondral damage but no ligament damage (McIlwraith 1992). However, the high incidence of tearing of the MPICL may be due to degeneration and weakening of the ligament as occurs in other intra-articular ligaments, such as the cruciate ligament, of other species (Vasseur, Pool et al. 1985). A histopathological study by Whitton *et al.*, (1999) of the MPICL showed a marked loss of normal ligamentous collagen architecture i.e. collagen fibre bundles were disorganised and poorly aligned, in all the MPICLs from treadmill trained adult horses examined compared to the ligaments from treadmill trained horses less than 12 months of age suggesting a degenerative change with age (Whitton and Rose 1999). Additionally, a high prevalence of MPICL tearing has been observed in horses with no history of joint disease (Whitton and Rose 1997), which suggests that damage to the MPICL may occur in isolation and is not necessarily associated with clinical signs of carpal joint disease.

To date, limited information exists on the biomechanical and biochemical properties of the MPICL, especially from clinically normal TB racehorses in racing and race-training.

A biomechanical study by Whitton *et al.*, (1997), provided information on the role of the palmar intercarpal ligaments in resisting dorsal displacement of the proximal row of carpal bones (Whitton and Rose 1997) but studies on the structural and material properties of the MPICL are lacking, particularly on comparing the mechanical properties with other ligament parameters such as ECM metabolism.

Additionally, as mentioned in Chapter One, a high incidence of tearing of the MPICL occurs predominantly within the dorsolateral (DL) branch and to a lesser extent the dorsomedial (DM) branch. The mechanisms for this are unclear, but Firth *et al.*, (1991) have suggested that this part of the MPICL might be more susceptible to longitudinal stress because, in electron microscopic sections of the DL and DM branches of the MPICL, the mean and range of collagen fibril diameter was found to be smaller in the DL than in the DM branch (Firth, Deanne et al. 1991). However, since this publication, the majority of research has been concerned with the MPICL as a whole and specific reference to the DL or DM branches has seldom been made.

Therefore the aims of this study were:

- To design a device and develop a protocol suitable for biomechanical testing of the bone-ligament-bone complex.
- To determine any differences in the structural, material and biochemical properties of the DL and DM branches of the MPICL from specimens subjected to racing and race-training compared to those from horses that have not raced, so as to assess if the stress applied to the carpal joint during high-intensity exercise is contributing to ligament failure.
- To ascertain any relationship with age and the structural, material and biochemical properties of the branches.
- To determine any differences in the structural, material and biochemical properties between pathological and non-pathological DL and DM branches.
- To determine any differences between the DL and DM branches.
- To determine if there is any relationship between the mechanical properties of the DL and DM branches and the biochemical properties, so

as to assess if changes in the collagen composition of the ligament are contributing to the failure/ rupture of the ligament.

4.2 MATERIALS AND METHODS

4.2.1 Ligament Biomechanics

4.2.1.1 Equine Samples

The biomechanical properties of the DL and DM branches were measured in the right and left carpi of raced and non-raced equine cadaver samples. Euthanasia was for reasons unrelated to orthopaedic injury or disease in both groups of horses. Table 4.1 shows the mean age and gender of the horses included in the study.

	Raced				Non-raced			
	DL		DM		DL		DM	
	Right	Left	Right	Left	Right	Left	Right	Left
Number of Equine Carpi (n)	8	7	7	6	7	12	10	7
Mean Age (years)	5	6	5	4	9	12	12	9
Number of Females	1	0	1	1	2	4	2	2
Number of Males	7	7	6	5	5	8	8	5

Table 4.1: Details of the horses used in the joint biomechanics study.

4.2.1.2 Ligament Pathology Grading

Directly after euthanasia the right and left carpi were opened to expose the midcarpal joint. The degree of ligament pathology/tearing of the DL and DM branches of the MPICL was graded using a scoring system adapted from Kannegieter *et al.*, (1993) (Kannegieter and Colgan 1993) (refer to Table 4.2). The intact midcarpal joint was subsequently frozen at –20°C until required.

Score	Ligament Damage
0	No apparent ligament damage
1	Mild damage with rupture, fraying or stretching of a small number of fibres
2	Up to 1/3 damage
3	1/3 to 2/3 damage
4	Complete rupture of ligament

Table 4.2: Scoring system used for the assessment of the degree of ligament damage. Adapted from Kannegieter *et al.*, (1993).

4.2.1.3 Cadaver Joint Preparation

Prior to mechanical testing, the midcarpal joints were thawed to room temperature. During all biomechanical testing the joints were kept as hydrated as possible by wrapping them in water soaked paper towel.

To allow independent analysis of the DL and DM branches, all soft tissue surrounding the MPICL was removed, with care taken not to damage or stress the MPICL. The PL and PM branches of the MPICL were carefully cut at their insertion points into the Cr, C2 and C3, and removed. In order to test the DL and DM branches in isolation, and dependent upon the branch was to be tested, the following dissections were performed:

- Biomechanical testing of the DL branch – This required severing the DM branch, which is attached to the Cr and C2, at its insertion point into the C2. This resulted in an intact DL branch attached to its respective carpal bones i.e. bone (Cr)-ligament (DL)-bone (C3).
- Biomechanical testing of the DM branch - This required severing the DL branch, which is attached to the Cr and C3, at its insertion point into the C3. This resulted in an intact DM branch attached to its respective carpal bones i.e. bone (Cr)-ligament (DM)-bone (C2).

4.2.1.4 Measurement of the Ligament Cross-Sectional Area

The cross-sectional area of the DL and DM branches is difficult to measure due to the irregular shape of the ligaments. Several methods have been used to determine the cross-sectional area of ligaments, including area micrometer (Allard, Thiry et al. 1979), mathematical calculations from width and depth measurements with callipers (Vasseur, Pool et al. 1985) and a gravimetric method measuring the weight and length of a ligament. However, these methods have limitations as often the apparatus comes into contact with the ligament. Race *et al.*, (1996) showed that the area micrometer method squeezes the cruciate ligament reducing the cross-sectional area recorded by up to 26% (Race and Amis 1996). Additionally, the mathematical calculations method is based on the assumption that the ligament has a regular cross-sectional shape and the gravimetric method relies on knowledge of a known specific weight. Other methods including travelling microscope, split shadow, laser telemetry and CT are non-contact but are still poor at recognising concavities of the ligament borders.

The method used in this study was that described by Race *et al.*, (1996), in which bone cement casts where utilised (Race and Amis 1996). This technique is non-destructive allowing measurement of the DL and DM branches whilst they were still attached to the carpal bones prior to failure testing.

Measurement protocol:

The midcarpal joint was placed in a clamping stand so that the entire ligament was fully elongated and exposed (refer to Figures 4.1 and 4.2). A thin 'pseudo-joint capsule' made of plasticine was moulded around the joint into which a silicon moulding was poured. Cold curing silicon rubber and its curing agent (Silcoset 105, Ambersil, UK) were mixed thoroughly at a ratio of 20ml silicon rubber to 6 drops of curing agent. This was then poured into the pseudo capsule until full, ensuing that it encapsulated the ligament (refer to Figure 4.3).

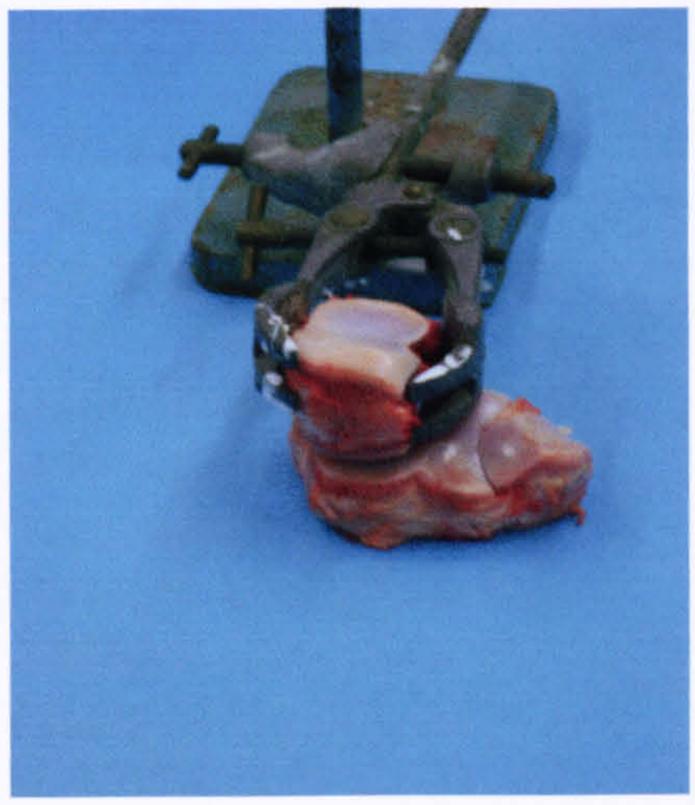


Figure 4.1: Photograph showing the clamping device used to secure the carpal bones prior to cross-sectional area quantification.

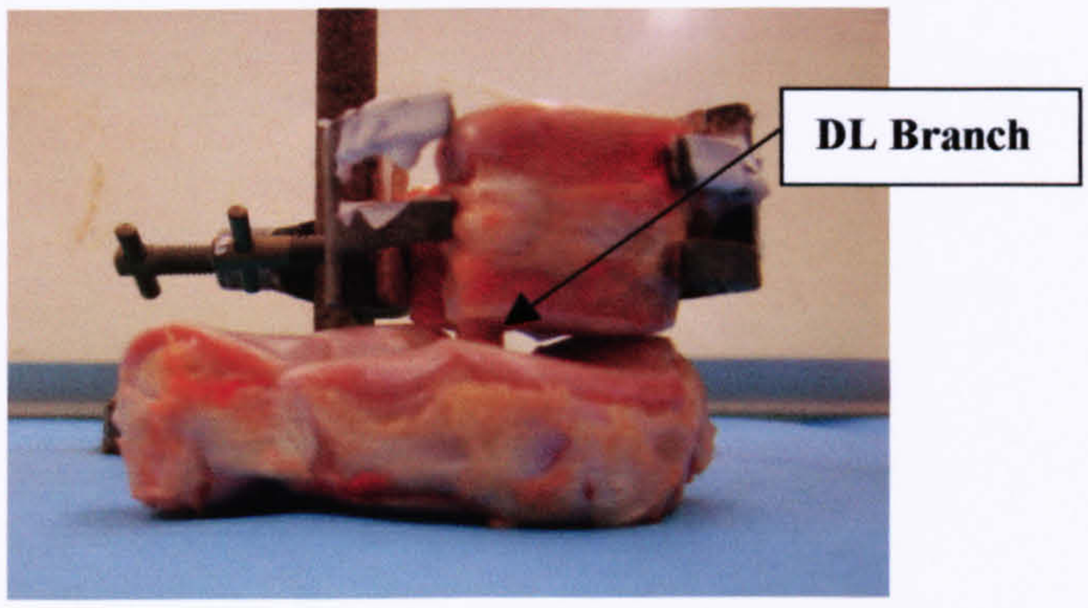


Figure 4.2: Photograph showing the exposed ligament prior to cross-sectional area quantification.

Figure 4.3: Photograph showing the pseudo-joint capsule and the silicon mould.

Once the silicon had set (approximately 1hr) the plasticine was removed and the hard silicon rubber mould was cut from the joint specimen with a scalpel blade. A vertical cut was made in the mould down one side, which was carefully deepened towards the ligament. The mould was peeled away from the intact ligament. The resultant mould possessed an impression of the Cr and C2/ C3 and a hole representing the DL or DM bundle (refer to Figure 4.4). The ligament impression was then filled with polymethylmethacrylate (PMMA) bone cement (Austenal Dental Simplex Rapid – self-curing, UK) at a mixture of 20ml curing agent to 23g powder. After 30 minutes, the silicon was cut away leaving a solid bone cement model of the ligament. The mid-point between the Cr and C2/C3 attachments was cut perpendicular to the axis loading of the ligament using a junior hacksaw. The section was then coloured with a black permanent marker and placed in a block of plasticine with a ruler in the field of view (refer to Figure 4.5). This was then photographed, scanned using Fotolook 2.05 and NIH Image 1.55 software and the cross-sectional area measured using 'Image Tool' (University of Ithaca).

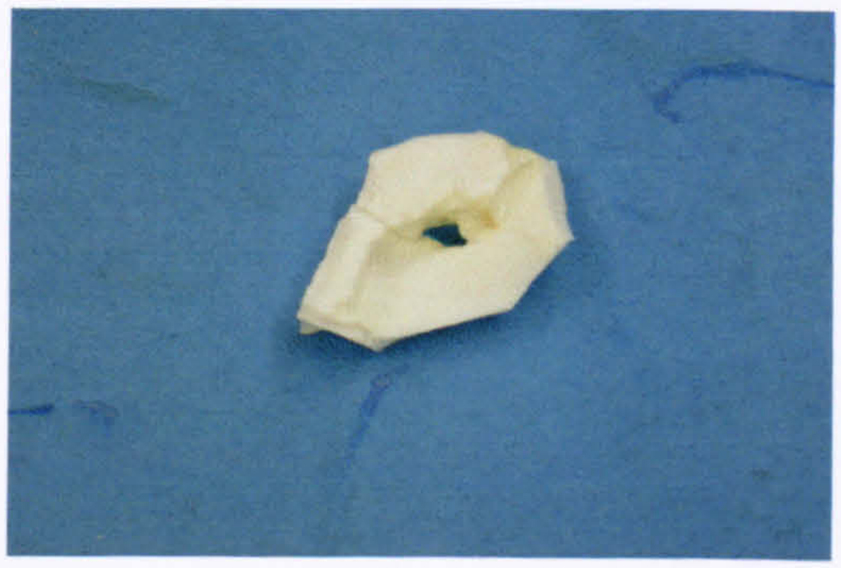


Figure 4.4: Photograph of the silicon mould with the hole representing the DL or DM bundle of the MPICL.

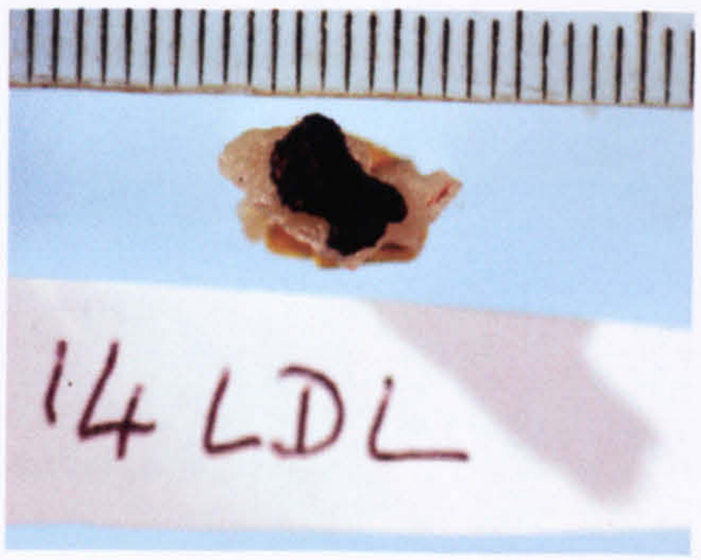


Figure 4.5: Photograph of the bone cement model of the cross-sectional area of the DL bundle alongside a mm ruler.

4.2.1.5 The Testing Apparatus

To date, limited information exists on the biomechanical testing of the MPICL and specifically the DL and DM branches, hence apparatus suitable for holding the bone-ligament-bone complex in the materials testing machine during tensile strength testing had to be specifically designed (refer to Figure 4.6). The angle at which the DL and DM branches of the MPICL reside within the midcarpal joint *in vivo* is complex (Whitton, McCarthy et al. 1997). Due to this practical implication, the apparatus did not allow testing to directly mimic the physiological conditions experienced *in vivo*, but biomechanically tests the ligament at a 90° angle. Importantly, however, this did allow the structural properties of the DL and DM branches to be determined.

Joint attachment to the testing apparatus:

Subsequent to obtaining the cross-sectional area of the ligament the bone-ligament-bone complex was attached to the aluminium testing apparatus. Prior to attachment, two holes of approximately 3cm distance were drilled into the C3 in a cranial to caudal plane, and two holes drilled approximately 2cm apart into the Cr in a lateromedial plane. Stainless steel rods were used to secure the Cr and C3 in the device (refer to Figure 4.6).

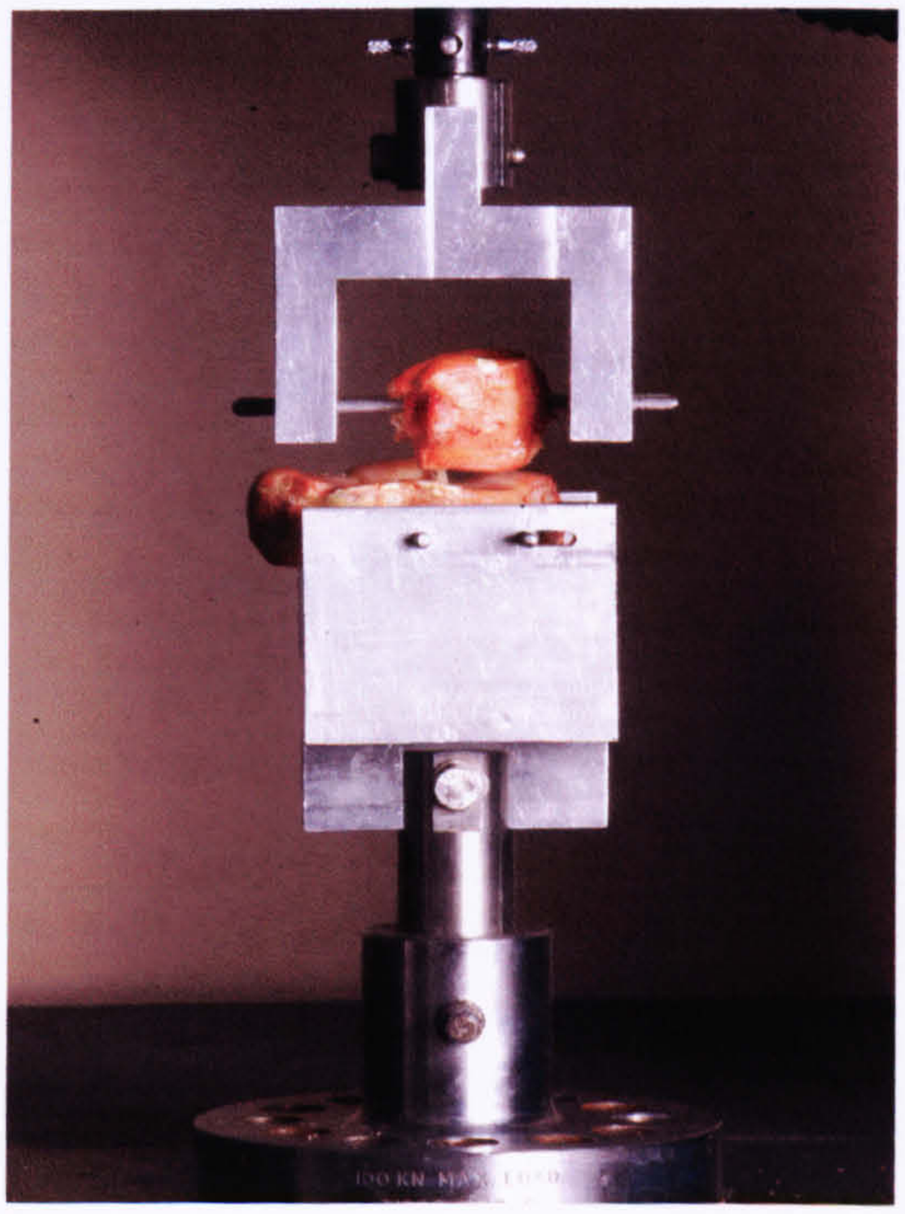


Figure 4.6: Photograph of the bone-ligament-bone complex attached to the specifically made aluminium testing apparatus.

4.2.1.6 Tensile Strength Testing

The DL and DM branches of the MPICL were tensile strength tested at a 90° angle in an Instron material testing system fitted with a 10kN load cell, at a test speed of 25mm/min to ligament failure (refer to Figure 4.7). Prior to testing it was ensured that the carpal bones had no contact with each other, to avoid interference with the results. This resulted in the slight straightening of the ligament, however, care was taken to ensure that the ligament was not under tensile load at this point.

A graph plotter and computer, both attached to the Instron machine, recorded the load-deformation curve of each specimen (refer to Figure 4.7).

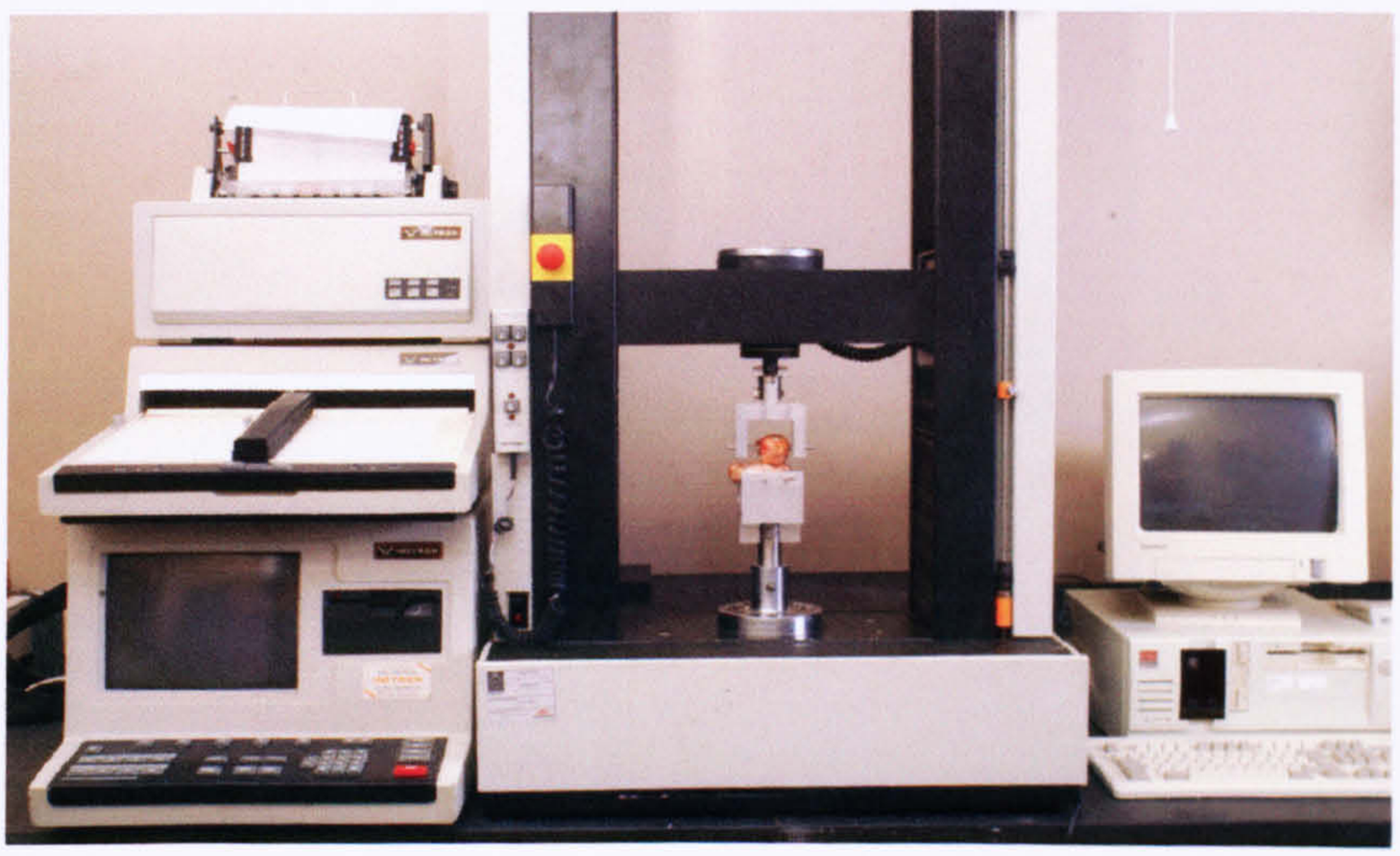


Figure 4.7: A photograph of an equine midcarpal joint mounted in the mechanical testing system and the equipment used during testing.

4.2.2 Ligament Collagen Metabolism

Details of the materials and solutions used in the following methods are listed in Appendices Four and Five respectively.

4.2.2.1 Equine Samples

The biochemical properties were additionally obtained on the left and right DL and DM branches of the MPICL. The details of the horses (left and right carpi) used in this part of the study are shown in Table 4.3.

	Raced				Non-raced			
	No Equine Samples (n)	Age (years)	No Females	No Males	No Equine Samples (n)	Age (years)	No Females	No Males
X-Links:								
DL	11	7	0	11	11	10	3	8
DM	10	7	1	9	12	9	4	8
Collagen Content:								
DL	11	7	0	11	11	10	3	8
DM	10	7	1	9	12	9	4	8
PICP:								
DL	11	7	0	11	9	11	3	6
DM	11	6	1	10	13	11	4	9
MMP:								
DL	12	7	1	11	20	11	6	14
DM	12	7	1	11	20	11	6	14
TIMP:								
DL	6	8	0	6	9	12	2	7
DM	8	6	1	7	8	11	3	5
DSC:								
DL	6	7	0	6	6	11	1	5
DM	7	7	1	6	6	11	1	5

Table 4.3: Details of the horses used in the biochemistry study (age= mean years).

4.2.2.2 Biochemical and Thermal Analyses

The methods used to quantify the various biochemical and thermal properties of the DL and DM branches of the MPICL are described elsewhere:

- Cross-link quantification – refer to Chapter Three, section 3.1.2.2.2.
- Hydroxyproline quantification (collagen content) - refer to Chapter Three, section 3.1.2.2.3.
- PICP content - refer to Chapter Three, section 3.1.2.2.5.
- MMP-2 and -9 quantification - refer to Chapter Three, section 3.1.3.1.1.
- TIMP-2 quantification - refer to Chapter Three, section 3.1.3.1.2.
- Thermal Analysis (DSC) - refer to Chapter Three, section 3.1.4.2.3.

Collagen II 3/4C_{short} Assay

As previously mentioned, a commercially available two-step competitive immunoassay (Ibex Diagnostics, USA) has been developed to quantify the amount of COL2-3/4C_{short} generated by the cleavage of types I and II collagens by collagenases. There is a lack of information on the cross-reactivity of the antibody to equine type I bone collagen, and the ability of this antibody to cross-react with equine type I ligament collagen has additionally not been documented. Hence part of this study was to investigate the use of this assay to quantify cleaved type I collagen in equine ligament extract.

The amount of COL2-3/4C_{short} epitope (µg/ml) was quantified in the ligament (DL and DM) extract of 3 non-raced and 6 raced horses, using the method previously described (refer to Chapter Three, section 3.1.3.2.6).

4.2.2.3 Statistical Analysis

Statistical analysis was performed using SPSS v11.5 (SPSS Inc. USA) and Graphpad Prism v3.0 (Graphpad Software Inc. USA).

Gender

Any relationships between gender and the biomechanical and collagen biochemical properties of the DL and DM branches of the ligament were investigated on a random selection of the parameters quantified i.e. the collagen content, the ultimate load at

failure, and the energy absorbed during testing. Depending upon the distribution of the data a parametric test (unpaired t-test) or non-parametric test (Mann-Whitney test) was used to determine any differences. Statistical significance was set at the level of $p<0.05$.

Right and Left Differences (applies to the biochemical data only)

Collagen biochemical data for the right and left DL and DM branches was obtained, hence a paired t-test was performed between the right and left values to ascertain any carpal joint asymmetry. However, the average value of the right and left was calculated and used in the subsequent results section since this represents the value of the individual horse.

Age and Comparisons Between Raced and Non-raced Horses

A correlation with age was investigated for all the biomechanical and biochemical variables quantified to determine any relationship with age (raced and non-raced horses data was pooled). Where a correlation was identified, univariate analysis of variance, using age as a covariate, was used to correct for the difference in the age of the horses. Statistical significance was set at the level of $p<0.05$. If there was no significant correlation or covariation with age, and dependent upon the distribution of the data, a parametric test (unpaired t-test) or non-parametric test (Mann-Whitney test) was used to determine differences between the raced and non-raced horses, the DL and DM branches and pathological and non-pathological specimens.

Ligament Biomechanics and Biochemistry Relationships

Dependent upon the distribution of the data, a parametric test (Pearson) or non-parametric test (Spearman) was used to determine any correlation in the biomechanical and biochemical properties of the DL and DM branches of the MPICL.

4.3. RESULTS

4.3.1 Ligament Biomechanics

4.3.1.1 Ligament Pathology

Previous studies have suggested that the four branches known to comprise the MPICL are not always present (Whitton and Rose 1997). In this study it was noted that while the four branches were always visible, occasionally it was difficult to discriminate between the two palmar branches of the MPICL, however the DL and DM branches were always easily observed and distinguishable.

a) Ligament pathology score

The degree of ligament pathology in the DL and DM branches of the raced and non-raced horses used within the biomechanics study is shown in Table 4.4 (the total number of carpi i.e. right plus left is given). For the ligament score of each individual horse see Appendix Two.

Ligament tearing in both the raced and non-raced horses was more prevalent in the DL bundle of the MPICL compared to the DM bundle; 47% (16 out of 34) of the carpal joints (raced and non-raced pooled) had grade 1 or above ligament pathology in the DL bundle compared to 7% (2 out of 30) in the DM bundle.

Ligament pathology in the DL bundle was greater in the raced compared to the non-raced carpi with 67% (10 out of 15) of the racehorses having grade 1 or above DL ligament pathology, compared to 31.5% (6 out of 19) of the non-racehorses having grade 1 or above. This was not evident in the DM branch. Complete rupture of both the DL or DM branches was not observed (grade 4).

Grade	Raced		Non-Raced	
	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)
0	5 (33.5%)	13 (100%)	13 (67%)	15 (88%)
1	5 (33.5%)	0 (0%)	2 (11%)	2 (12%)
2	3 (20%)	0 (0%)	2 (11%)	0 (0%)
3	2 (13%)	0 (0%)	2 (11%)	0 (0%)
4	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 4.4: The degree of ligament pathology in the DL and DM branches of the MPICL subjected to biomechanical testing in the raced and non-raced horses.

b) Correlation with age

The ages of the horses varied from 2 to 22 years. The degree of ligament pathology did not significantly correlate with age in either the right (r^2= -0.001, p= 0.90) or left (r^2= 0.02, p= 0.51) DL or the right (r^2= 0.43, p= 0.10) or left (r^2= 0.46, p= 0.10) DM branches.

4.3.1.2 Ligament Cross-sectional Area Measurements (mm^2)

a) Correlation with age

The ligament cross-sectional area measurements did not significantly correlate with age in either the right (r^2= -0.01, p= 0.68) or left (r^2= -0.09, p= 0.26) DL or the right (r^2= 0.001, p= 0.91) or left (r^2= -0.01, p= 0.73) DM branches.

b) Comparisons between raced and non-raced horses

The cross-sectional areas of the right and left DL and DM branches of the raced and non-raced horses are shown in Table 4.5. There was no significant difference in the cross-sectional area measurements between the raced and non-raced right or left DL and DM branches.

	DL Cross-sectional Area (Mean +/- SEM)	P Value	DM Cross-sectional Area (Mean +/- SEM)	P Value
Raced Right	11.08 +/- 2.54	0.12	11.15 +/- 1.15	0.27
Non-raced Right	17.78 +/- 3.31		14.45 +/- 2.28	
Raced Left	9.41 +/- 2.04	0.76	14.79 +/- 2.21	0.15
Non-raced Left	10.05 +/- 1.02		18.59 +/- 1.33	

Table 4.5: The cross-sectional area (mean mm^2 +/- SEM) and p value of the right and left DL and DM branches of raced and non-raced horses.

c) Comparisons between the DL and DM branches

The cross-sectional area was significantly greater in the left DM compared to the left DL (p=0.0002). There was no significant difference between the right DL and right DM (p=0.64) (refer to Figure 4.8). The raced and non-raced data was pooled.

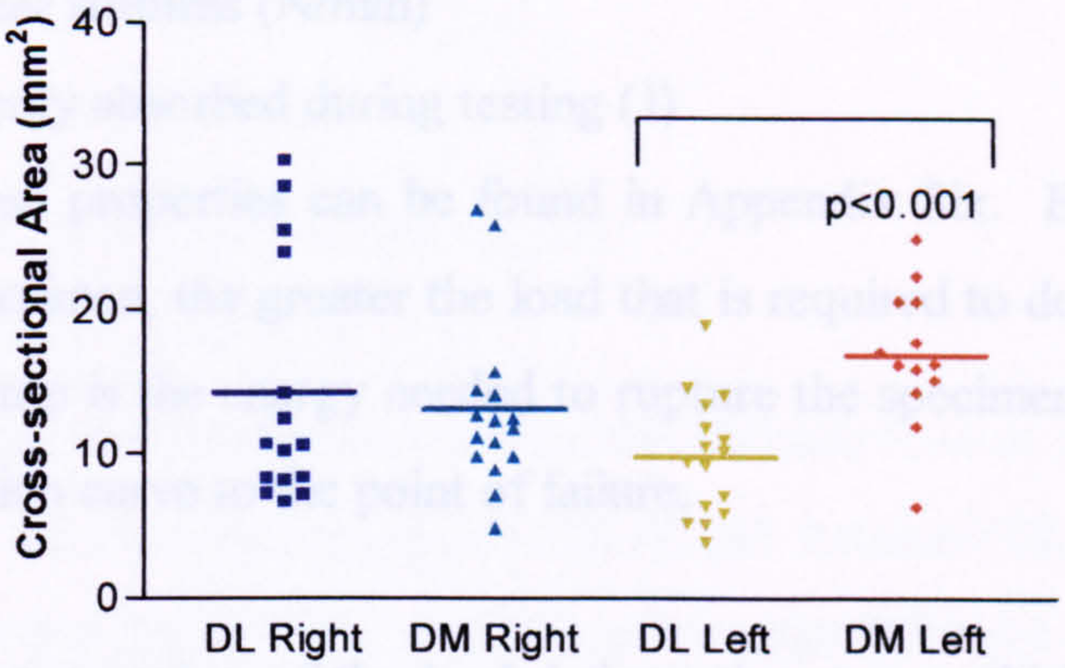


Figure 4.8: The cross-sectional area of the left and right DL and DM branches.

d) Comparisons between pathological and non-pathological samples

Pathological samples refer to those DL and DM branches graded 1 or above, and the non-pathological refer to those graded 0.

Due to a lack of pathological DM ligament samples, only the DL ligament pathological and non-pathological data was analysed. In addition, due to the lack of pathological non-racehorse data, the raced and non-raced pathological, and the raced and non-raced non-pathological data, was pooled and analysed. NB: This applies to all subsequent analysis.

There was no significant difference between the right (p=0.44) pathological (15.82+/-3.1) and non-pathological (12.37+/-3.09) cross-sectional areas or the left (p=0.41) pathological (10.77+/-1.8) and non-pathological (8.98+/-1.14) cross-sectional areas of the DL bundle.

4.3.1.3 The Structural Properties of the Ligaments

Figure 4.9 shows a typical load-deformation curve produced by the Instron chart recorder for a DL or DM bundle of the MPICL. This curve provides the following structural (mechanical) information on the ligament:

- The ultimate load at failure (N)
- The linear stiffness (N/mm)
- The energy absorbed during testing (J)

Definitions of these properties can be found in Appendix Six. Briefly, the greater the stiffness of the specimen, the greater the load that is required to deform it by unit length. The energy at failure is the energy needed to rupture the specimen and is the area under the load-deformation curve to the point of failure.

Unfortunately the toe region of the load-deformation curve which is thought to reflect fibre straightening and the reduction of crimp at a microscopic level (Frank, Amiel et al. 1985) was unable to be quantified since the ligament had to be straightened to avoid contact of the carpal bones prior to testing and as such the toe region measurements differed extensively from specimen to specimen, being immeasurable in a large proportion of the load-deformation curves.

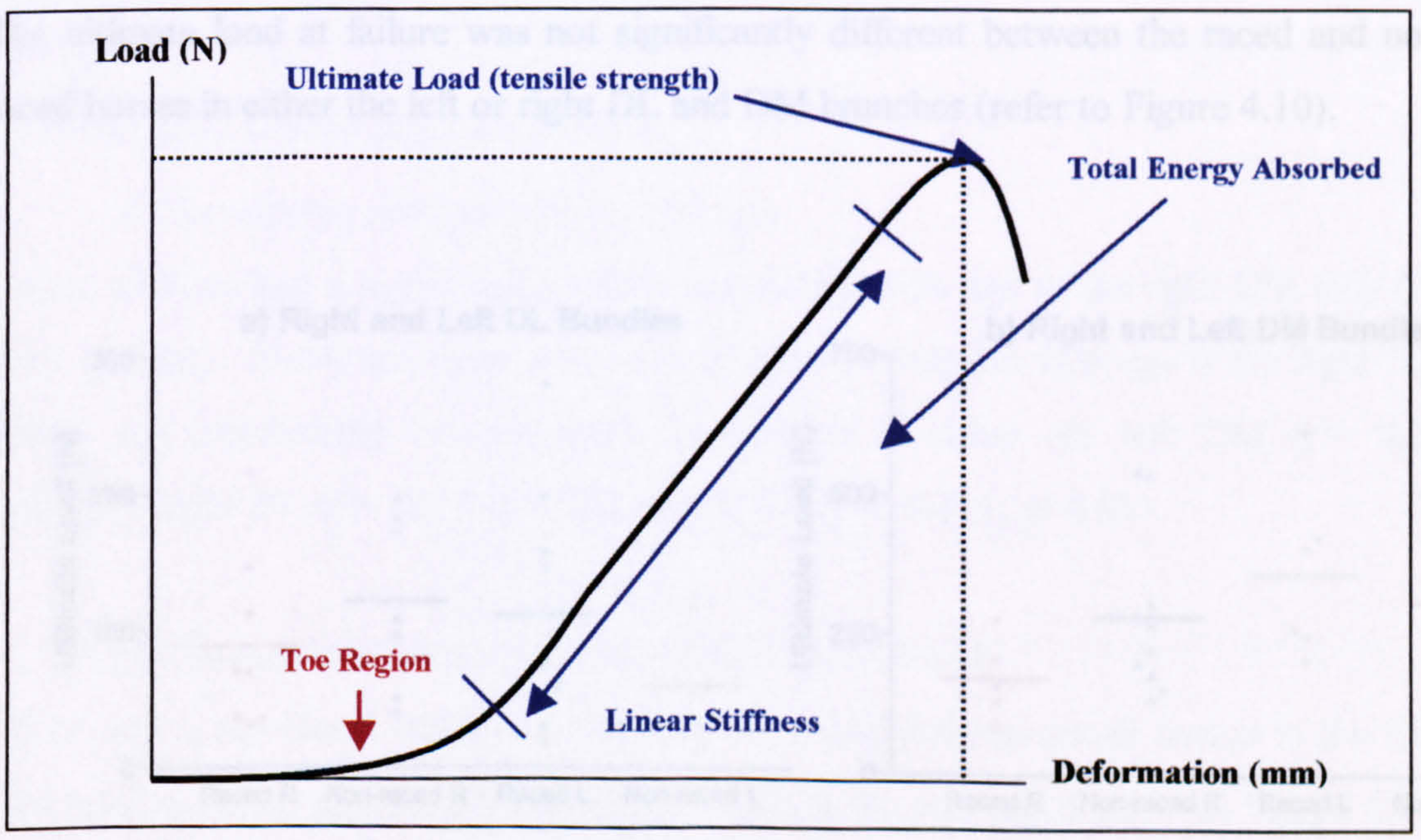


Figure 4.9: A schematic diagram of a typical load-deformation curve for a DL and DM bundle of the MPICL.

Failure of ligament at its insertion points has been documented to occur during biomechanical testing, with femoral and tibial avulsions for example, being observed during tensile strength testing of the canine CCL (Wingfield, Amis et al. 2000). During the tensile strength testing of the DL and DM branches of the MPICL in this study it was noted that failure occurred mid-way between the Cr and C2/C3 attachments.

4.3.1.3.1 Ultimate Load at Failure (N):

a) Influence of gender

There was no significant difference between the ultimate load at failure in the right plus left DL ($p=0.98$) between males (99.98+/15.3) and females (88.3+/-18.34) and in the DM ($p=0.85$) between males (328.2+/-59.2) and females (284+/-67.4).

b) Correlation with age

There was no significant correlation in the ultimate load to failure and age in either the right ($r^2= -0.18$, $p= 0.11$) or left ($r^2= -0.02$, $p= 0.88$) DL and the right ($r^2= 0.04$, $p= 0.45$) or left ($r^2= -0.29$, $p= 0.06$) DM branches.

c) Comparisons between raced and non-raced horses

The ultimate load at failure was not significantly different between the raced and non-raced horses in either the left or right DL and DM branches (refer to Figure 4.10).

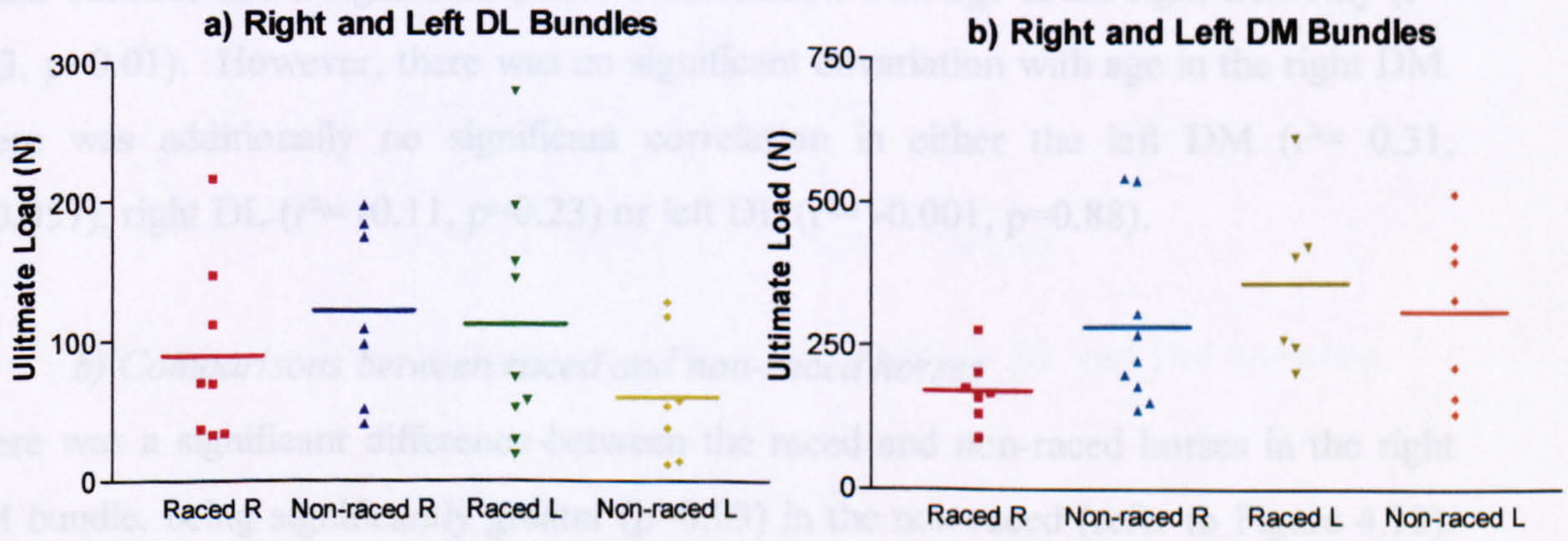


Figure 4.10: The ultimate load at failure in the left and right a) DL and b) DM branches of raced and non-raced horses. NB: R= right DL/DM. L= left DL/DM.

d) Comparisons between the DL and DM branches

The ultimate load to failure was significantly higher in both the right and left DM compared to the right and left DL (refer to Figure 4.11).

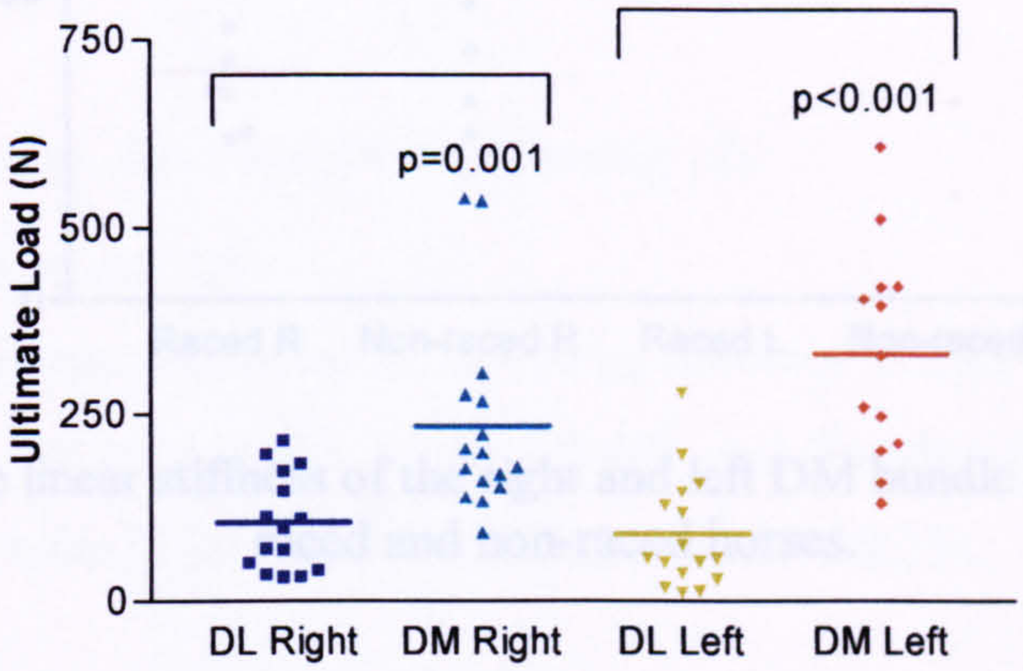


Figure 4.11: The ultimate load at failure between the left and right DL and DM branches.

e) Comparisons between pathological and non-pathological samples

There was no significant difference in the ultimate load at failure between the right (p=0.93) pathological (104.1+/-24.1) and non-pathological (107.1+/-24.4) or the left (p=0.66) pathological (80.09+/-26.1) and non-pathological (97.1+/-27.6) DL branches.

4.3.1.3.2 Linear Stiffness (N/mm):

a) Correlation and covariation with age

Linear stiffness had a significant positive correlation with age in the right DM only (r^2= 0.03, p=0.01). However, there was no significant covariation with age in the right DM. There was additionally no significant correlation in either the left DM (r^2= 0.31, p=0.057), right DL (r^2= -0.11, p=0.23) or left DL (r^2= -0.001, p=0.88).

b) Comparisons between raced and non-raced horses

There was a significant difference between the raced and non-raced horses in the right DM bundle, being significantly greater (p=0.03) in the non-raced (refer to Figure 4.12). There was no significant difference in the left DM or right and left DL.

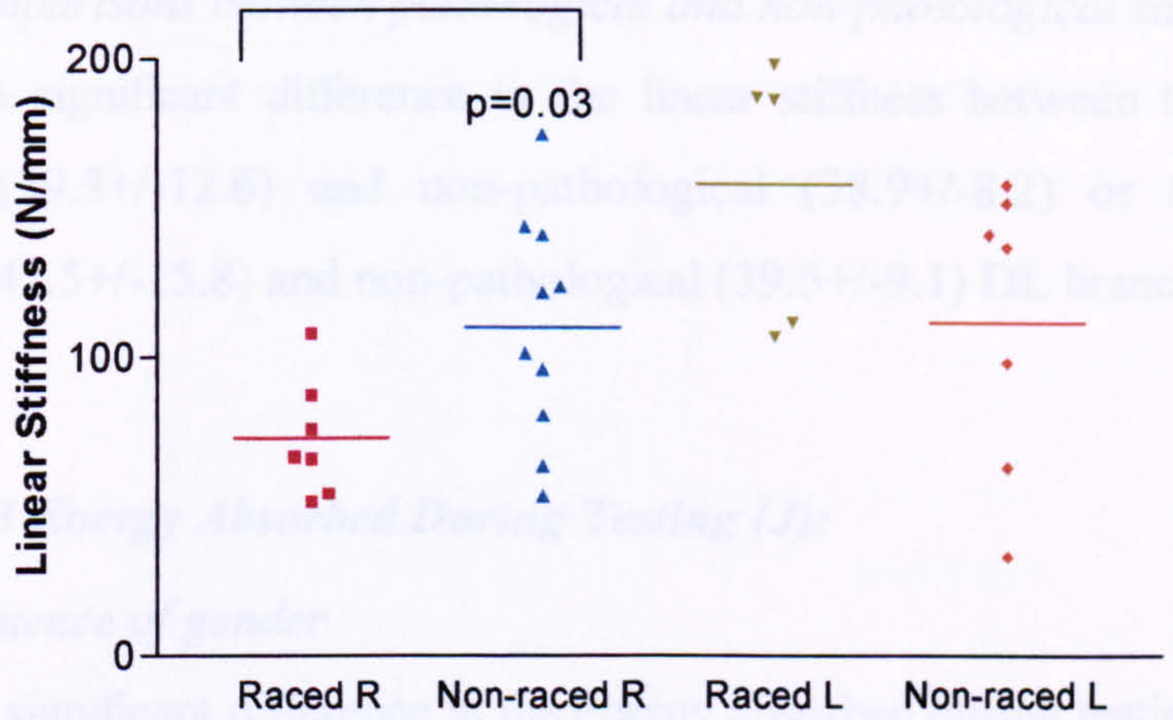


Figure 4.12: The linear stiffness of the right and left DM bundle of the MPICL in the raced and non-raced horses.

c) Comparisons between the DL and DM branches

Linear stiffness was significantly higher in the both the right and left DM compared to the right and left DL (refer to Figure 4.13).

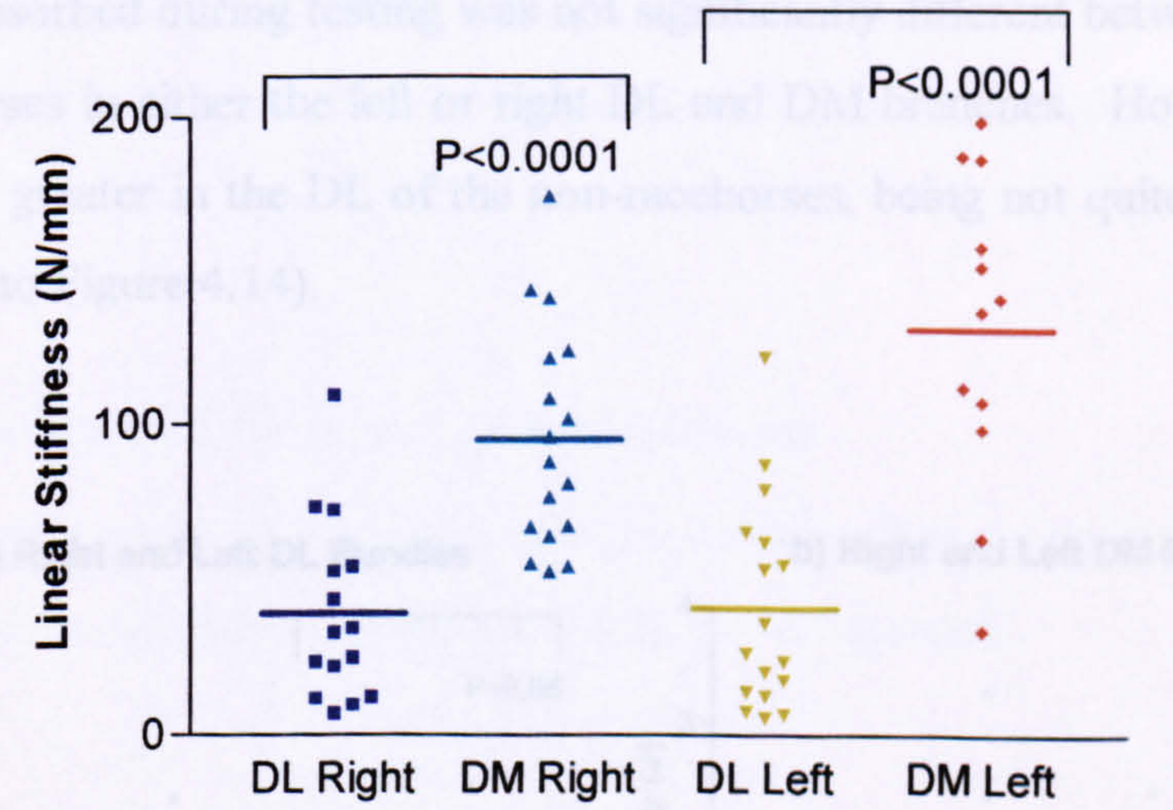


Figure 4.13: The linear stiffness between the left and right DL and DM branches.

d) Comparisons between pathological and non-pathological samples

There was no significant difference in the linear stiffness between the right (p=0.98) pathological (39.3+/-12.6) and non-pathological (38.9+/-8.2) or the left (p=0.86) pathological (42.5+/-15.8) and non-pathological (39.5+/-9.1) DL branches.

4.3.1.3.3 Energy Absorbed During Testing (J):

a) Influence of gender

There was no significant difference in the energy absorbed during testing in the right plus left DL (p=0.68) between males (0.38+/-0.06) and females (0.44+/-0.07) and DM (p=0.52) between males (1.2+/-0.17) and females (0.14+/-0.25).

b) Correlation with age

There was no significant correlation in the energy absorbed during testing and age in either the right (r^2= -0.03, p= 0.49) or left (r^2= -0.03, p= 0.49) DL and the right (r^2= -0.04, p= 0.42) or left (r^2= -0.05, p= 0.43) DM branches.

c) Comparisons between raced and non-raced horses

The energy absorbed during testing was not significantly different between the raced and non-raced horses in either the left or right DL and DM branches. However, the energy absorbed was greater in the DL of the non-racehorses, being not quite significant in the left DL (refer to Figure 4.14).

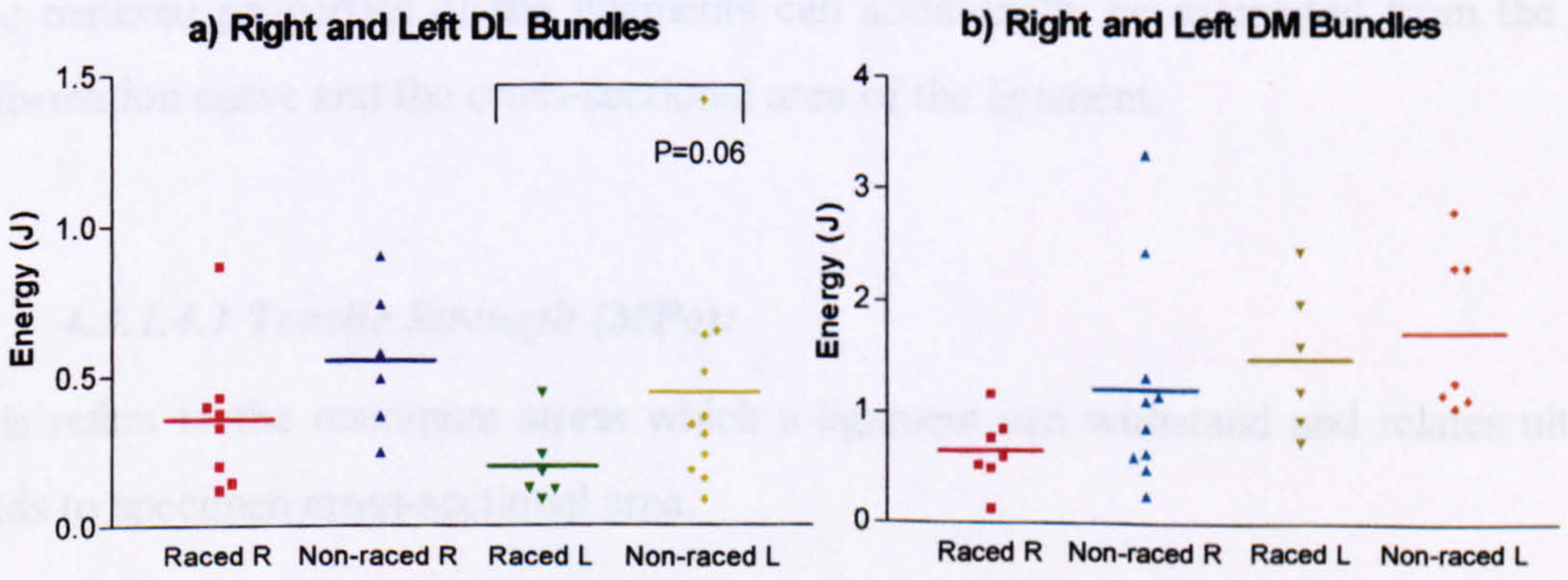


Figure 4.14: The energy absorbed during testing in the left and right DL and DM branches of raced and non-raced horses.

d) Comparisons between the DL and DM branches

The energy absorbed during testing was significantly greater in the both the right and left DM compared to the right and left DL (refer to Figure 4.15).

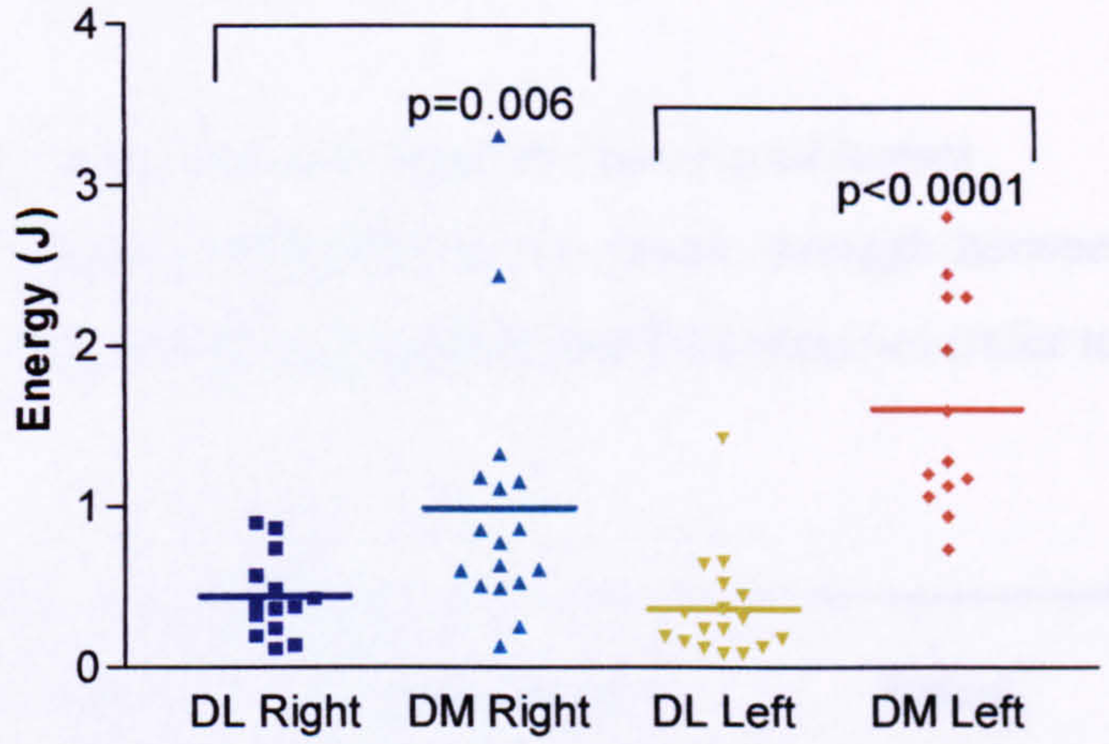


Figure 4.15: The energy absorbed during testing in the left and right DL and DM branches.

e) Comparison between pathological and non-pathological samples

There was no significant difference in the energy absorbed during testing between the right (p=0.86) pathological (0.43+/-0.09) and non-pathological (0.45+/-0.1) or the left (p=0.73) pathological (0.28+/-0.07) and non-pathological (0.41+/-0.12) DL branches.

4.3.1.4 The Material Properties of the Ligaments

The material properties of the ligaments can additionally be calculated from the load-deformation curve and the cross-sectional area of the ligament.

4.3.1.4.1 Tensile Strength (MPa):

This refers to the maximum stress which a ligament can withstand and relates ultimate loads to specimen cross-sectional area.

a) Correlation and covariation with age

The ultimate tensile strength had a significant negative correlation with age in the left DM only (r^2= 0.44 p=0.01). There was no significant covariation with age in the left DM. There was additionally no significant correlation in the either the right DM (r^2= 0.095, p=0.28), right DL (r^2= -0.14, p=0.16) or left DL (r^2= 0.06, p=0.35).

b) Comparisons between raced and non-raced horses

There was no significant difference in the tensile strength between the raced and non-raced horses in either the left or right DL and DM branches (refer to Table 4.6).

Tensile Strength (MPa)	DL		DM	
	Raced	Non-raced	Raced	Non-raced
Right	8.36+/-1.6	7.89+/-1.9	16.32+/-1.7	20.38+/-3.1
Left	7.04+/-1.8	11.75+/-3.1	25.42+/-3.7	16.61+/-2.6

Table 4.6: The tensile strength of the DL and DM of the raced and non-raced horses (values are mean MPa +/- SEM).

c) Comparisons between the DL and DM branches

The tensile strength was significantly greater in the right and left DM compared to the right and left DL (refer to Figure 4.16).

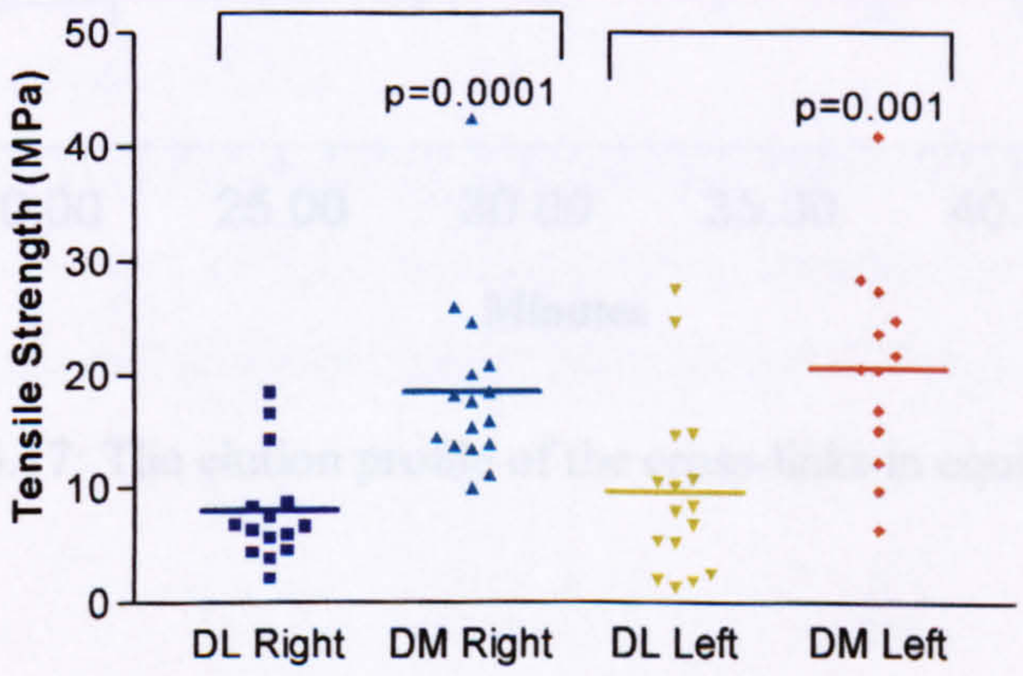


Figure 4.16: The tensile strength of the left and right DL and DM branches.

d) Comparison between pathological an non-pathological samples

There was no significant difference in the tensile strength between the right (p=0.31) pathological (6.95+/-1.2) and non-pathological (9.51+/-2.1) or the left (p=0.22) pathological (6.97+/-1.77) and non-pathological (11.8+/-3.0) DL branches.

4.3.2 Ligament Collagen Metabolism

4.3.2.1 Markers of Collagen Synthesis

4.3.2.1.1 Collagen Cross-links (mole/mole collagen):

The cross-link elution profile for equine ligament is illustrated in Figure 4.17. The cross-link types were the same in both the DL and DM branches of the MPICL, with the immature, diHLNL and HLNL forms, and the mature, Lys-Pyr, HL-Pyr and HHL forms being present. The elastin derived cross-linked amino acids, Desmosine (Des) and Iso-Desmosine (Ides), were also visible on the ligament elution profiles.

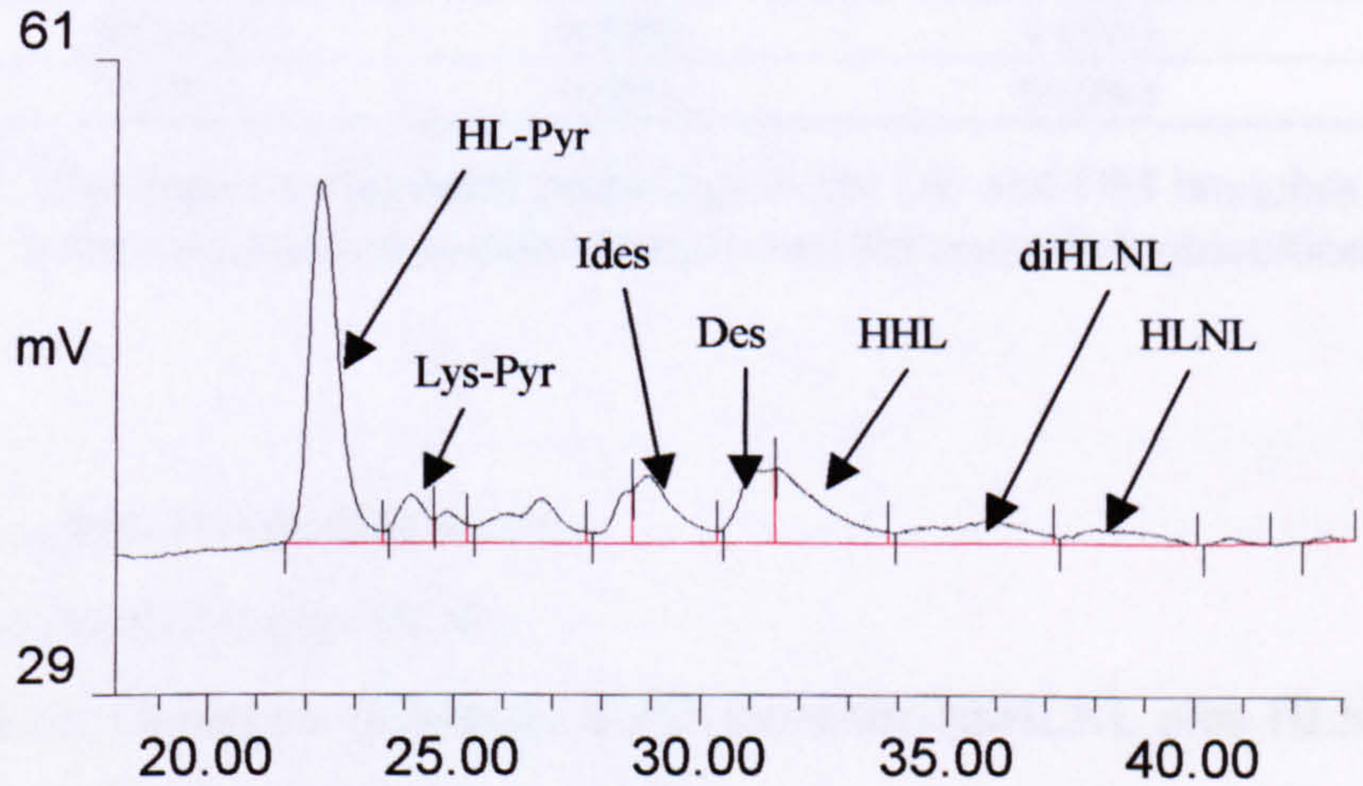


Figure 4.17: The elution profile of the cross-links in equine ligament.

a) Ligament pathology score

Table 4.7 shows the degree of ligament pathology in the DL and DM branches of the raced and non-raced horses used to quantify the collagen cross-link content (the total number of carpi i.e. right plus left is given). The degree of ligament tearing in both the raced and non-raced horses is more prevalent in the DL bundle of the MPICL compared to the DM bundle; 61% (22 out of 36) of the carpal joints had grade 1 or above ligament pathology in the DL bundle compared to 12% (5 out of 41) in the DM bundle.

Ligament pathology in the DL bundle was greater in the racehorses compared to the non-racehorses carpi with 80% (16 out of 20) of the racehorses having grade 1 or above DL ligament pathology, compared to 37.5% (6 out of 16) of the non-racehorses having grade 1 or above. This was not evident in the DM branch.

Grade	Raced		Non-Raced	
	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)
0	4 (20%)	17 (94%)	10 (63%)	19 (83%)
1	4 (20%)	1 (6%)	3 (19%)	3 (13%)
2	7 (35%)	0 (0%)	2 (13%)	1 (4%)
3	4 (20%)	0 (0%)	1 (5%)	0 (0%)
4	1 (5%)	0 (0%)	0 (0%)	0 (0%)

Table 4.7: The degree of ligament pathology in the DL and DM branches of the MPICL in the raced and non-raced horses used for cross-link quantification.

b) Left and right differences

i) Immature (diHLNL plus HLNL)

There was no significant difference in the immature (diHLNL plus HLNL) and mature (Lys-Pyr plus HL-Pyr and HHL) cross-link content between the right and left DL and DM in both the raced and non-raced horses (see Appendix Three).

c) Correlation with age

The ages of the horses varied from 3 to 17 years. The collagen cross-links (diHLNL, HLNL, Lys-Pyr, HL-Pyr and HHL) did not significantly correlate with age in either the DL or DM branches (refer to Table 4.8).

Layer	Linear (Pearson) Correlation (r^2)	P Value
diHLNL		
DL	-0.007	0.71
DM	-0.03	0.40
HLNL		
DL	-0.01	0.66
DM	-0.001	0.85
HL-Pyr		
DL	-0.02	0.54
DM	-0.0006	0.91
Lys-Pyr		
DL	-0.002	0.83
DM	-0.006	0.72
HHL		
DL	+0.12	0.12
DM	-0.06	0.28

Table 4.8: Correlation coefficients of the cross-link content and age in the DL and DM.

d) Comparisons between raced and non-raced horses

The cross-links were increased, often significantly, in the DL and DM branches from the raced compared to the non-raced horses (refer to Table 4.9). Additionally, the immature (diHLNL plus HLNL) to mature (HL-Pyr plus Lys-Pyr) ratio was significantly greater in the DL and DM of the raced compared to the non-raced horses (refer to Figure 4.18).

	DL		DM	
	Raced	**Non-raced**	**Raced**	**Non-raced**
DiHLNL:	0.03+/-0.01**	0.002+/-0.0008	0.02+-/0.006*	0.003+/-0.0009
HLNL:	0.02+/-0.004	0.006+/-0.002	0.02+/-0.005*	0.005+/-0.002
HL-Pyr:	0.36+/-0.04*	0.24+/-0.02	0.42+/-0.05*	0.29+/-0.03
Lys-Pyr:	0.04+/-0.01	0.02+/-0.005	0.06+/-0.01*	0.03+/-0.005
HHL:	0.07+/-0.01	0.07+/-0.02	0.07+/-0.02	0.03+/-0.006

Table 4.9: The cross-link content in the DL and DM of the raced and non-raced horses (values are mean moles/moles collagen +/- SEM. Significant results= *($P<0.05$), **($P<0.01$)).

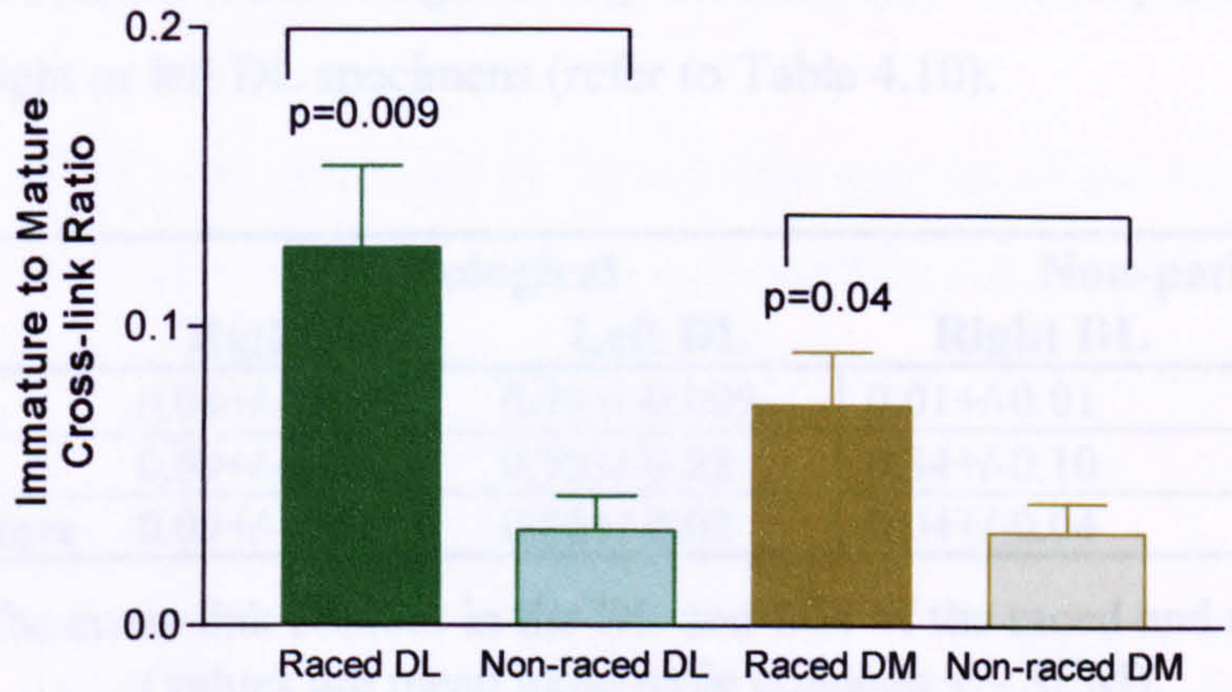


Figure 4.18: The immature to mature cross-link ratio in the DL and DM branches of the raced and non-raced horses.

e) Comparisons between the DL and DM branches

There was no significant difference in the level of collagen cross-links (diHLNL, HLNL, Lys-Pyr, HL-Pyr and HHL) between the DL or DM branches, however, the immature to mature ratio was greater in the DL compared to the DM (refer to Figure 4.19).

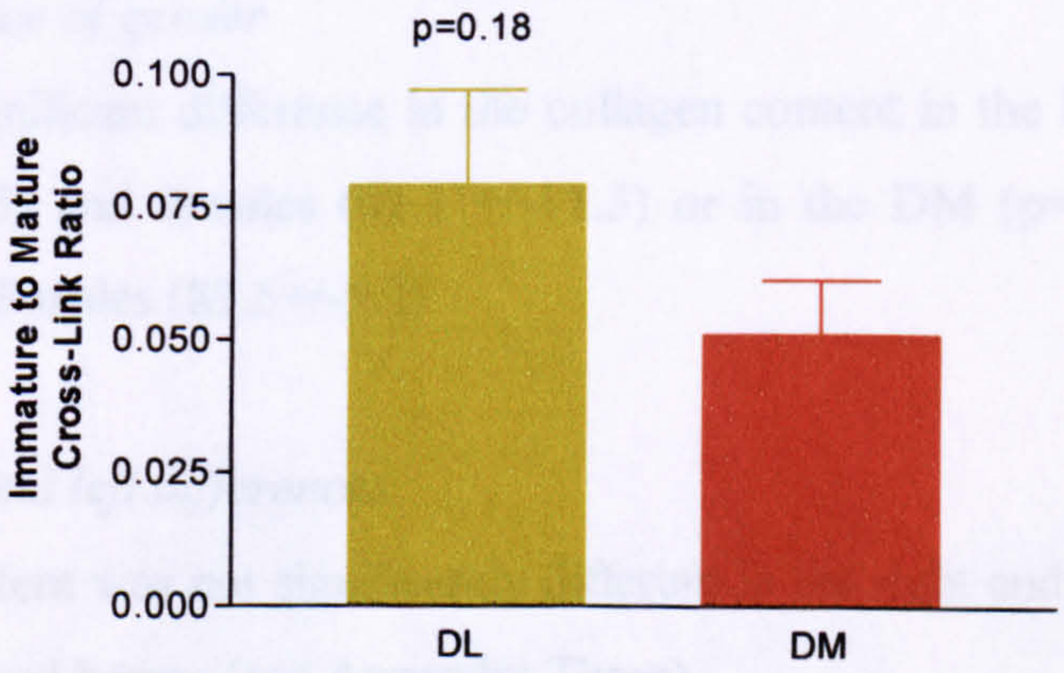


Figure 4.19: The immature to mature cross-link ratio in the DL and DM branches.

f) Comparison between pathological and non-pathological samples

As for section 4.3.1.2 only the DL pathological and non-pathological data has been analysed and the raced and non-raced pathological and non-pathological data has been pooled.

The cross-link content was not significantly different between the pathological and non-pathological right or left DL specimens (refer to Table 4.10).

	Pathological		Non-pathological	
	Right DL	**Left DL**	**Right DL**	**Left DL**
Immature	0.04+/-0.02	0.03+/-0.009	0.01+/-0.01	0.02+/-0.007
Mature	0.59+/-0.14	0.75+/-0.22	0.34+/-0.10	0.54+/-0.14
Immature:Mature	0.09+/-0.04	0.08+/-0.02	0.04+/-0.04	0.05+/-0.02

Table 4.10: The cross-link content in the DL and DM of the raced and non-raced horses (values are mean mole/mole collagen +/- SEM).

4.3.2.1.2 Collagen Content (% dry weight):

a) Ligament pathology score

The collagen content was quantified on the same samples used for cross-link quantification, therefore, refer to section 4.3.2.2.1 for details on the ligament pathology score.

b) Influence of gender

There was no significant difference in the collagen content in the DL (p=0.25) between males (75.6+/-2.5) and females (61.1 +/-11.3) or in the DM (p=0.17) between males (71.7+/-3.6) and females (83.5+/-9.2).

c) Right and left differences

The collagen content was not significantly different in the right and left DL or DM of the raced and non-raced horses (see Appendix Three).

d) Correlation with age

The ages of the horses varied from 3 to 17 years. The collagen content did not significantly correlate with age in either the DL (r^2= -0.03, p= 0.39) or DM (r^2= -0.02, p= 0.49) branches of the MPICL.

e) Comparisons between raced and non-raced horses and the DL and DM branches

There was no significant difference in the collagen content of the DL (p=0.88) or DM (p=0.161) branches between the raced and non-raced horses. Additionally there was no significant difference between the DL and DM branches (p=0.713) (refer to Table 4.11).

	DL	DM
Raced	73.2+/-2.1	68.8+/-2.9
Non-raced	72.3+/-5.1	79.1+/-5.8
DL vs. DM	72.7+/-2.7	74.4+/-3.5

Table 4.11: The collagen content in the DL and DM of the raced and non-raced horses (values are mean %d dry weight +/- SEM).

f) Comparison between pathological and non-pathological samples

The collagen content was significantly higher in the right DL pathological specimens compared to the right DL non-pathological specimens (refer to Figure 4.20). However, there was no significant difference in the left DL.

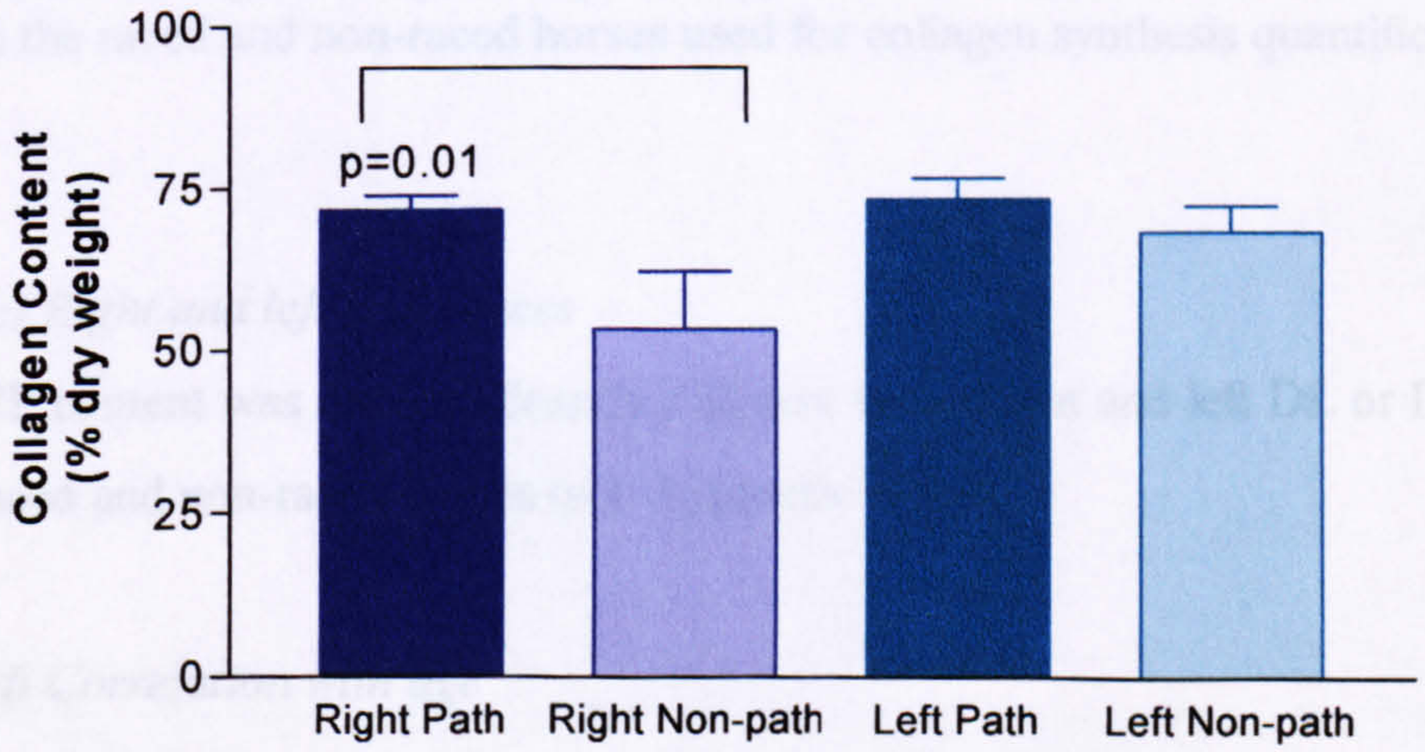


Figure 4.20: The collagen content in the right DL bundle of the pathological and non-pathological specimens.

4.3.2.1.3 Collagen Type I Synthesis (PICP) (µg/l):

a) Ligament pathology score

The degree of ligament pathology in the DL and DM branches of the raced and non-raced horses used to quantify the levels of type I collagen synthesis are shown in Table 4.12. Again, the degree of ligament tearing in both the raced and non-raced horses is greater in the DL bundle of the MPICL compared to the DM bundle; 64% (18 out of 28) of the carpi had grade 1 or above ligament pathology in the DL bundle compared to 7.5% (3 out of 40) in the DM bundle.

Ligament pathology in the DL bundle was greater in the raced compared to the non-raced carpi with 80% (16 out of 20) of the racehorses having grade 1 or above DL ligament pathology, compared to 40% (4 out of 10) of the non-racehorses having grade 1 or above. This was not evident in the DM branch.

Grade	Raced		Non-Raced	
	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)
0	4 (22%)	19 (95%)	6 (60%)	18 (90%)
1	4 (22%)	1 (5%)	3 (30%)	2 (10%)
2	6 (33%)	0 (0%)	0 (0%)	0 (0%)
3	4 (22%)	0 (0%)	1 (10%)	0 (0%)
4	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 4.12: The degree of ligament pathology in the DL and DM branches of the MPICL in the raced and non-raced horses used for collagen synthesis quantification.

c) Right and left differences

The PICP content was not significantly different in the right and left DL or DM branches of the raced and non-raced horses (see Appendix Three).

d) Correlation with age

The ages of the horses varied from 3 to 22 years. The PICP content did not significantly correlate with age in either the DL (r^2= -0.02, p=0.56) or DM (r^2= -0.01, p=0.57) branches.

e) Comparisons between raced and non-raced horses and the DL and DM branches

There was no significant difference in the PICP content of the DL (p=0.29) or DM (p=0.35) between the raced and non-raced horses. Additionally there was no significant difference between the DL and DM branches (p=0.33) (refer to Table 4.13).

	DL	DM
Raced	119.4+/-25.4	93.2+/-28.6
Non-raced	210+/-64.7	184+/-57.1
DL vs. DM	160.2+/-33	142.7+/-34.2

Table 4.13: The collagen synthesis content in the DL and DM of the raced and non-raced horses (values are mean µg/l +/- SEM).

f) Comparison between pathological and non-pathological samples

Due to a lack of right non-pathological samples the right and left data was pooled. The PICP content was found to be significantly elevated in the non-pathological DL branches (refer to Figure 4.21).

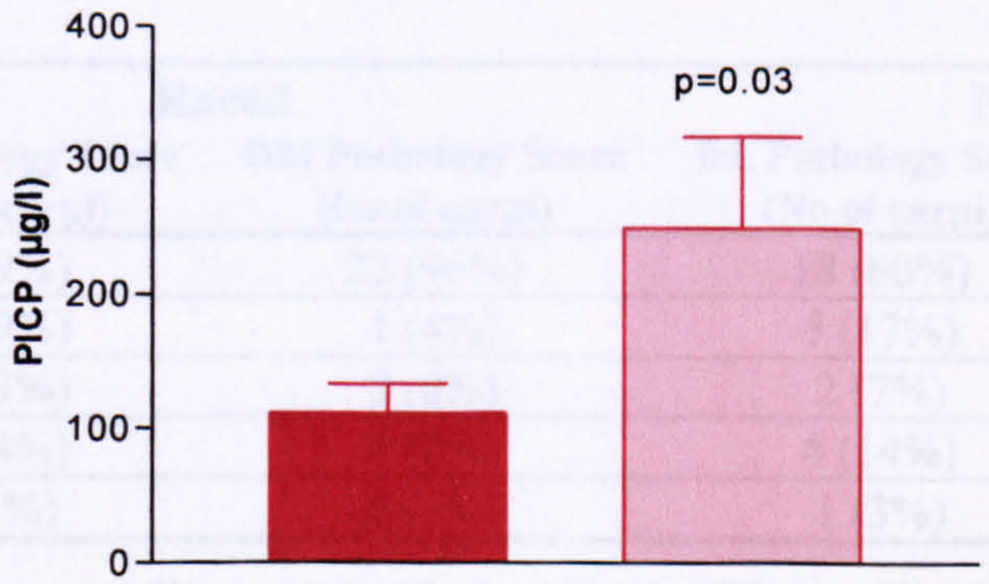


Figure 4.21: The PICP content in the pathological and non-pathological DL branches.

4.3.2.2. Markers of Ligament Collagen Degradation

4.3.2.2.1 Total MMP-2 and –9 (% std):

Gelatin zymography showed the presence of the pro and active forms of MMP-2 and MMP-9, in the DL and DM branches of the MPICL. As for the results expressed in Chapter 3, section 3.1.3.2, the total MMP-2 and-9 levels are represented in the following results.

a) Ligament pathology score

Table 4.14 shows the degree of ligament pathology in the DL and DM branches of the raced and non-raced horses used to quantify MMP-2 and -9 expression. The degree of ligament tearing in both the raced and non-raced horses is greater in the DL branch of the MPICL compared to the DM branch; 56% (29 out of 51) of the carpi had grade 1 or above ligament pathology in the DL branch compared to 13% (7 out of 55) in the DM branch.

Ligament pathology in the DL branch was greater in the raced compared to the non-raced carpal joints with 78% (14 out of 18) of the racehorses having grade 1 or above DL ligament pathology, compared to 40% (12 out of 30) of the non-racehorses having grade 1 or above. This was not evident in the DM branch.

Grade	Raced		Non-Raced	
	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)
0	4 (19%)	22 (96%)	18 (60%)	26 (81%)
1	4 (19%)	1 (4%)	5 (17%)	5 (16%)
2	7 (33%)	0 (0%)	2 (7%)	1 (3%)
3	5 (24%)	0 (0%)	4 (14%)	0 (0%)
4	1 (5%)	0 (0%)	1 (3%)	0 (0%)

Table 4.14: The degree of ligament pathology in the DL and DM branches of the MPICL in the raced and non-raced horses used for quantification of the MMP-2 and-9 activity.

b) Right and left differences

There was no significant difference in the MMP-2 and MMP-9 expression between the right and left DL or DM in both the raced and non-raced horses (see Appendix Three).

c) Correlation with age

The ages of the horses varied from 3 to 22 years. MMP-2 and -9 levels did not significantly correlate with age in either the DL or DM branches (refer to Table 4.15)

Layer	Linear (Pearson) Correlation (r^2)	P Value
MMP-2		
DL	-0.0006	0.89
DM	-0.02	0.47
MMP-9		
DL	0.04	0.27
DM	0.06	0.20

Table 4.15: Correlation coefficients of the MMP expression and age in the DL and DM.

d) Comparisons between raced and non-raced horses and the DL and DM branches

There was no significant difference in the MMP-2 or MMP-9 levels in the DL or DM between the raced and non-raced horses (refer to Table 4.16). Additionally there was no significant difference in the MMP-2 and-9 expression between the DL and DM branches.

	DL		DM	
	Raced	**Non-raced**	**Raced**	**Non-raced**
MMP-2:	54.9+/-10.6	63.2+/-8.8	41.9+/-6.6	57.7+/-9.0
MMP-9:	29.5+/-5.5	23.9+/-2.6	23.1+/-5.3	28.7+/-4.4

Table 4.16: The MMP expression in the DL and DM of the raced and non-raced horses (Values are mean %std +/- SEM).

e) Comparison between pathological and non-pathological samples

The MMP-2 levels were not significantly different in the right DL (p=0.97) between pathological (68.65+/-12.6) and non-pathological (106.4+/-40.7) or in the left DL (p=0.53) between pathological (55.07+/-10.2) and non-pathological (46.93+/-8.1). Additionally, the MMP-9 levels were not significantly different in the right DL (p=0.71) between pathological (23.01+/-4.4) and non-pathological (32.38+/-12.1) or in the left DL (p=0.62) between pathological (25.4+/-3.3) and non-pathological (27.81+/-3.3).

4.3.2.2.2 TIMP-2 (% std):

TIMP-1 expression could not be quantified due to its presence in very low levels, hence only the levels of TIMP-2 are given in the following results.

a) Ligament pathology score

Table 4.17 shows the degree of ligament pathology in the DL and DM branches of the raced and non-raced horses used to quantify TIMP-2 levels. The degree of ligament tearing in both the raced and non-raced horses is greater in the DL branch of the MPICL compared to the DM branch; 60% (9 out of 15) of the carpi had grade 1 or above ligament pathology in the DL branch compared to 5% (1 out of 19) in the DM branch.

There was no difference in the level of ligament pathology in the DL branch of the raced and non-raced carpi. Both the racehorse and non-racehorse DL branches had 50% (3 out of 6) grade 1 or above ligament pathology. This pattern was similar in the DM branch.

Grade	Raced		Non-Raced	
	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)
0	3 (50%)	11 (100%)	3 (33%)	7 (88%)
1	2 (33%)	0 (0%)	3 (33%)	1 (13%)
2	1 (17%)	0 (0%)	1 (11%)	0 (0%)
3	0 (0%)	0 (0%)	2 (22%)	0 (0%)
4	0 (0%)	0 (0%)	0 (3%)	0 (0%)

Table 4.17: The degree of ligament pathology in the DL and DM branches of the MPICL in the raced and non-raced horses used for quantification of the TIMP-2 activity.

b) Right and left differences

There was no significant difference in the TIMP-2 expression between the right and left DL or DM branches in both the raced and non-raced horses (see Appendix Three).

c) Correlation with age

The ages of the horses varied from 3 to 22 years. The level of TIMP-2 did not significantly correlate with age in either the DL (r^2= 0.007, p=0.75) or DM (r^2= -0.02, p=0.62) branches.

d) Comparisons between raced and non-raced horses and the DL and DM branches

There was no significant difference in the TIMP-2 expression in the DL (p=0.68) or DM (p=0.99) branches between the raced and non-raced horses. There was also no significant difference in the TIMP-2 expression between the DL and DM (p=0.37) branches (refer to Table 4.18).

	DL	DM
Raced	36.6+/-9.7	29.3+/-5.5
Non-raced	32.7+/-3.8	29.3+/-3.7
DL vs. DM	34.4+/-4.6	29.3+/-3.2
Path vs. Non-path	33.5+/-4.3	38.3+/-10.8

Table 4.18: The TIMP-2 expression in the DL and DM branches of the raced and non-raced horses (values are mean %std +/- SEM).

e) Comparison between pathological and non-pathological samples

Due to the lack of right non-pathological DL samples, the right and left data has been pooled. However, the levels of TIMP-2 were not significantly different between the pathological and non-pathological right and left DL specimens (refer to Table 4.18).

4.3.2.2.3 COL2-3/4C$_{short}$ Epitope (µg/ml):

The ELISA developed to quantify the amount of COL2-3/4C$_{short}$ generated by the cleavage of types I and II collagens by collagenases was found to cross-react with equine type I ligament collagen. Figure 4.22 shows the amount of COL2-3/4C$_{short}$ (µg/ml) in the DL and DM branches of the MPICL (raced and non-raced data was pooled). There was no significant difference between the branches.

Due to a lack of non-racehorse tissue, the amount of COL2-3/4C$_{short}$ epitope (µg/ml) could not be compared between the two groups of horses.

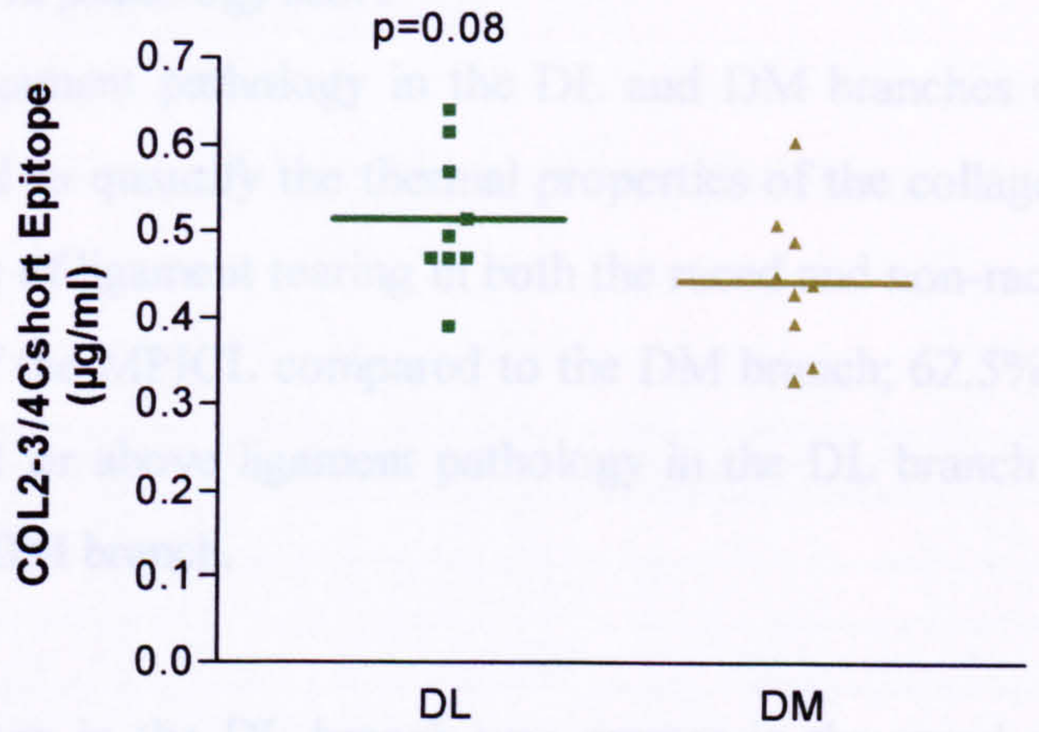


Figure 4.22: The amount of COL2-3/4C_{short} epitope (µg/ml) in the DL and DM branches.

4.3.2.3 The Thermal Properties of Collagen

A typical DSC thermogram of equine ligament is shown in Figure 4.23 (the thermograms do not differ within the DL and DM branches of the MPICL). The enthalpy (J/mg) and T_{max} (°C) obtained from the endotherm (see Chapter 3, section 3.1.4) are illustrated in the following results.

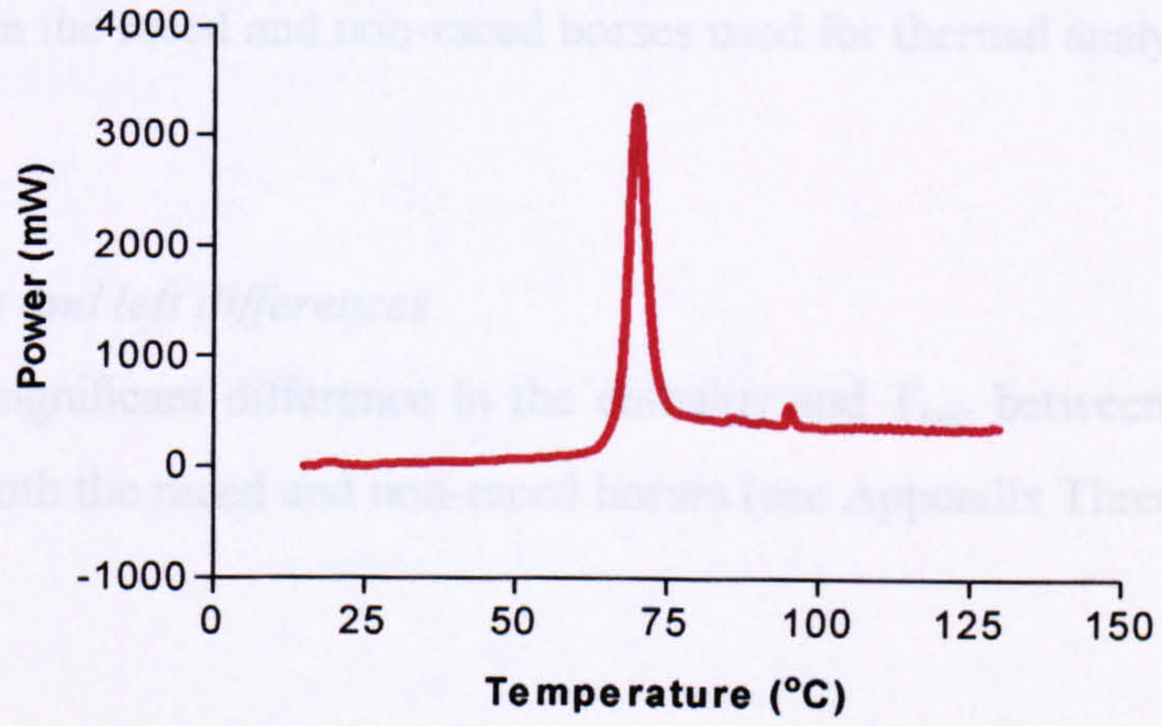


Figure 4.23: DSC thermogram of equine ligament.

a) Ligament pathology score

The degree of ligament pathology in the DL and DM branches of the raced and non-raced horses used to quantify the thermal properties of the collagen are shown in Table 4.19. The degree of ligament tearing in both the raced and non-raced horses is greater in the DL branch of the MPICL compared to the DM branch; 62.5% (15 out of 24) of the carpi had grade 1 or above ligament pathology in the DL branch compared to 15% (4 out of 26) in the DM branch.

Ligament pathology in the DL branch was greater in the raced compared to the non-raced carpi with 75% (9 out of 12) of the racehorses having grade 1 or above DL ligament pathology, compared to 50% (6 out of 12) of the non-racehorses having grade 1 or above. This was not evident for the DM branch.

Grade	Raced		Non-Raced	
	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)	DL Pathology Score (No of carpi)	DM Pathology Score (No of carpi)
0	3 (25%)	13 (93%)	6 (650%)	9 (75%)
1	3 (25%)	1 (7%)	2 (17%)	2 (17%)
2	2 (17%)	0 (0%)	2 (17%)	1 (8%)
3	3 (25%)	0 (0%)	2 (17%)	0 (0%)
4	1 (8%)	0 (0%)	0 (0%)	0 (0%)

Table 4.19: The degree of ligament pathology in the DL and DM branches of the MPICL in the raced and non-raced horses used for thermal analysis.

b) Right and left differences

There was no significant difference in the enthalpy and T_{max} between the right and left DL or DM in both the raced and non-raced horses (see Appendix Three).

c) Correlation with age

The ages of the horses varied from 4 to 17 years. The enthalpy and T_{max} did not significantly correlate with age in either the DL or DM branches (refer to Table 4.20).

Layer	Linear (Pearson) Correlation (r^2)	P Value
Enthalpy		
DL	-0.03	0.61
DM	-0.03	0.59
T_{max}		
DL	-0.17	0.18
DM	-0.49	0.08

Table 4.20: Correlation coefficients of the thermal characteristics and age in the DL and DM branches of the MPICL.

d) Comparisons between raced and non-raced horses and the DL and DM branches

The enthalpy of denaturation was significantly greater in the DM branch of the non-raced horses ($p=0.04$) compared to the DM branch of the raced horses (refer to Figure 4.24a). Additionally the T_{max} was higher in the DL and DM branches of the raced compared to the non-raced horses, being significantly greater in the DL branch ($p=0.005$) (refer to Figure 4.24b). There was no significant difference in the enthalpy or T_{max} between the DL and DM branches of the MPICL.

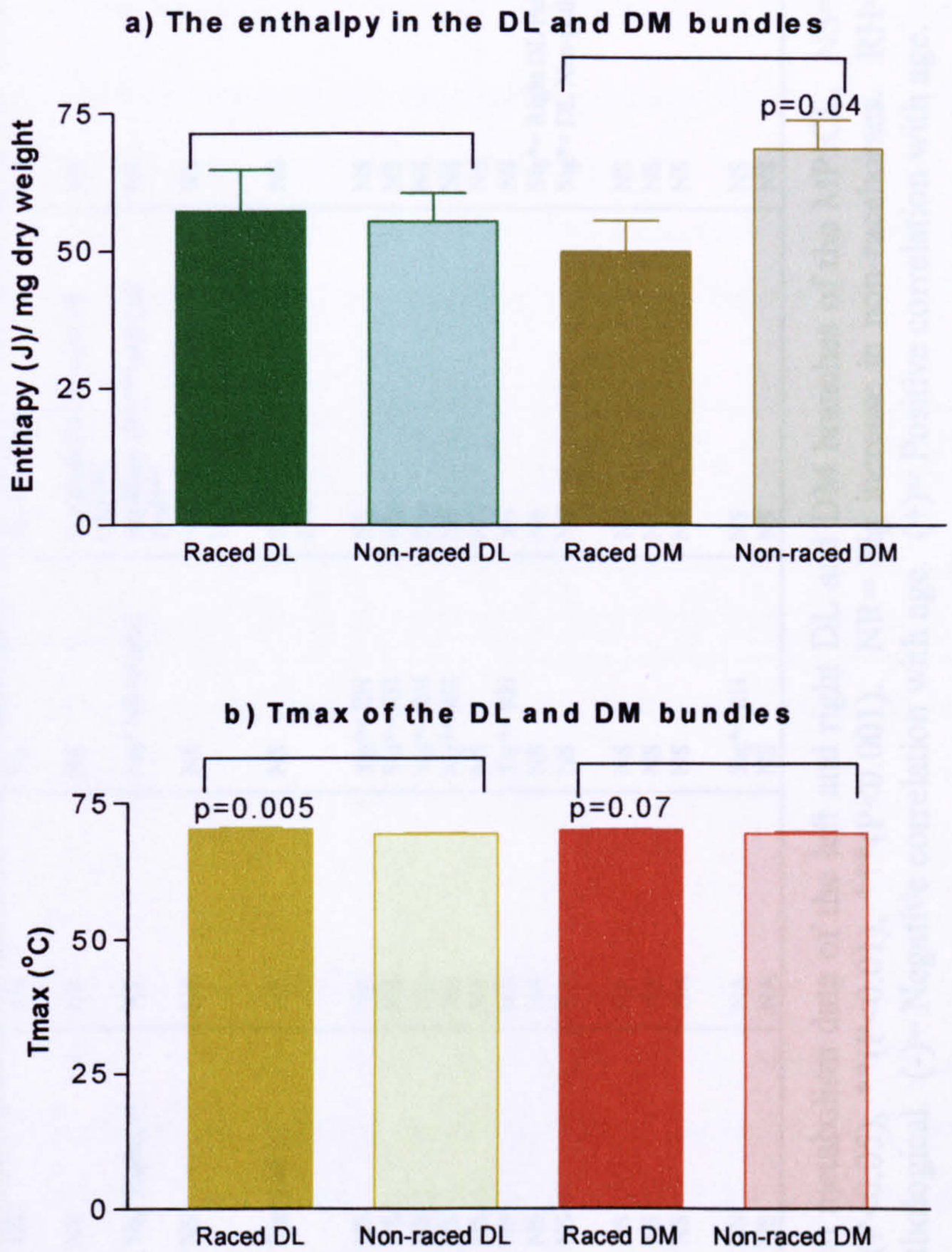


Figure 4.24: The enthalpy (a) and T_{max} (b) of the DL and DM branches of the raced and non-raced horses.

e) Comparison between pathological and non-pathological samples

The right and left data has been pooled due to the lack of right non-pathological specimens. There was no significant difference in the enthalpy (p=0.46) between the pathological (55.85+/-5.1) and non-pathological (49.79+/-5.8) DL samples or in the T_{max} (p=0.63) between the pathological (70.06+/-0.2) and non-pathological (69.87+/-0.1) DL samples.

Ligament Biomechanics and Collagen Metabolism	DL Branch			DM Branch			DL v DM	Path v Non-Path DL Branch
	Correlation with Age	Covariation with Age	Significant Difference RH & NR	Correlation with Age	Covariation with Age	Significant Difference RH&NR	Significant Difference	Significant Difference
Cross-Sectional Area	NS	NA	NS	NS	NA	NS	Sig left DM*	NS
Structural Properties:								
a) Ultimate Load	NS	NA	NS	NS	NA	NS	Sig Right DM** and Left DM***	NS
b) Linear Stiffness	NS	NA	NS	Sig* Right (+)	NS	Sig* NR (Right)	Sig Right DM*** and Left DM***	NS
c) Energy Absorbed	NS	NA	NS	NS	NA	NS	Sig Right DM*** and Left DM***	NS
Material Properties:								
a) Tensile Strength	NS	NA	NS	Sig* Left (-)	NS	NS	Sig Right DM*** and Left DM**	NS
Collagen Cross-Links:								
diHLNL	NS	NA	Sig**= RH	NS	NA	Sig*= RH	NS	NS
HLNL	NS	NA	NS	NS	NA	Sig*= RH	NS	NS
HL-Pyr	NS	NA	Sig*= RH	NS	NA	Sig*= RH	NS	NS
Lys-Pyr	NS	NA	NS	NS	NA	Sig*= RH	NS	NS
HHL	NS	NA	NS	NS	NA	NS	NS	NS
Immature: Mature	NS	NA	Sig**= RH	NS	NA	Sig*= RH	NS	NS
Collagen Content:	NS	NA	NS	NS	NA	NS	NS	Sig*= Right DL Path
PICP:	NS	NA	NS	NS	NA	NS	NS	Sig*= DL Non-path
MMP:								
MMP-2	NS	NA	NS	NS	NA	NS	NS	NS
MMP-9	NS	NA	NS	NS	NA	NS	NS	NS
TIMP-2:	NS	NA	NS	NS	NA	NS	NS	NS
DSC:								
Enthalpy	NS	NA	NS	NS	NA	Sig*= RH	NS	NS
T_{max}	NS	NA	Sig**= RH	NS	NA	NS	NS	NS

Table 4.21: Summary of the ligament biomechanics and collagen metabolism data of the left and right DL and DM branches of the MPICL. NS= Not significant. NA= Not applicable. Sig= Significant results= *($P<0.05$), **($P<0.01$), ***($P<0.001$). NR= Sig increase in non-racehorses. RH= Sig increase in racehorses. Path= Pathological. Non-path= Non-pathological. (-)= Negative correlation with age. (+)= Positive correlation with age.

4.3.3 Ligament Biomechanics and Biochemistry Relationships (correlations)

The DL and DM branches from the raced and non-raced horses that had been subjected to biomechanical testing and biochemical analysis, were used in the following investigation. The average right and left DL data from each horse was used, as was the average right and left DM data from each horse (refer to Table 4.22).

	Raced		Non-raced	
	DL Average Right and Left	**DM** Average Right and Left	**DL** Average Right and Left	**DM** Average Right and Left
Number of Horses (n)	9	8	14	14
Mean Age (years)	7	6	11	10

Table 4.22: Details of the horses subjected to biomechanical testing and biochemical analysis.

4.3.3.1 The Dorsolateral Branch

Table 4.23 shows the correlation coefficients of the various biomechanical and biochemical properties quantified in the DL branch of the raced and non-raced horses. A correlation was only found to exist between the MMP-2 levels and linear stiffness in the non-racehorses, with the linear stiffness decreasing as the MMP-2 levels increase.

	Raced DL				Non-Raced DL			
Parameter	**Ultimate Load**	**Tensile Strength**	**Linear Stiffness**	**Energy Absorbed**	**Ultimate Load**	**Tensile Strength**	**Linear Stiffness**	**Energy Absorbed**
Immature Cross-links	-0.42	-0.71	-0.52	-0.11	+0.20	+0.14	+0.05	+0.15
Mature Cross-links	+0.03	+0.37	-0.003	-0.02	-0.09	-0.08	-0.07	+0.003
Collagen Content	+0.27	-0.06	+0.057	+0.34	-0.05	-0.02	-0.01	-0.17
Total MMP-2	-0.0005	+0.36	-0.0004	-0.10	-0.12	-0.23	-0.37*	-0.28
DSC T_{max}	+0.11	+0.52	+0.03	-0.0003	-0.09	-0.77	-0.54	-0.02

Table 4.23: Correlation coefficients of the various biomechanical and biochemical properties quantified in the DL branch of raced and non-raced horses.
Significant results *($P<0.05$).

4.3.3.2 Dorsomedial Branch

Table 4.24 shows the correlation coefficients of the various biomechanical and biochemical properties quantified in the DM branch of the raced and non-raced horses. A correlation was found to exist between the collagen content and the ultimate tensile strength again in the non-racehorses, with the tensile strength increasing as the collagen content increases.

	Raced DM				Non-Raced DM			
Parameter	**Ultimate Load**	**Tensile Strength**	**Linear Stiffness**	**Energy Absorbed**	**Ultimate Load**	**Tensile Strength**	**Linear Stiffness**	**Energy Absorbed**
Immature Cross-links	-0.02	-0.16	+0.18	+0.029	-0.02	+0.001	+0.01	-0.0006
Mature Cross-links	+0.54	-0.01	+0.02	-0.44	-0.008	-0.19	-0.001	+0.05
Collagen Content	-0.00004	-0.01	-0.06	+0.18	+0.06	+0.43*	+0.16	-0.09
Total MMP-2	-0.006	-0.28	-0.24	+0.07	+0.19	+0.14	+0.07	-0.04
DSC T_{max}	-0.01	+0.04	+0.14	-0.06	+0.04	-0.053	+0.02	+0.13

Table 4.24: Correlation coefficients of the various biomechanical and biochemical properties quantified in the DM branch of raced and non-raced horses. Significant results *($P<0.05$).

4.4 DISCUSSION

Biomechanics Testing Protocol

A protocol for tensile strength testing the DL and DM branches of the MPICL has not been previously described, hence apparatus suitable for biomechanically testing the ligament to failure had to be developed. Due to the complexity of the anatomical positioning of the DL and DM within the midcarpal joint, the ligament was loaded directly perpendicular to the load cell (see Figures 4.7 and 4.8), which appeared appropriate to obtain details on the structural and material properties of the DL and DM branches.

Mechanical and Biochemical Properties

Racing and race-training does appear to cause a greater incidence of tearing and pathology in the MPICL, specifically in the DL branch of the MPICL, the branch which has been previously documented to be most prone to tearing (McIlwraith 1992; Phillips and Wright 1994; Whitton, McCarthy et al. 1997). These results are dissimilar to the results in the study by Whitton *et al.*, (1997), in which no significant difference was found in the severity of tearing between racing and non-racing Standardbreds and TBs (Whitton and Rose 1997). However, that study failed to grade the individual branches composing the MPICL, and hence a specific comparison with the level of tearing in the DL and DM branches could not be made. It is important to note however, that only mild tearing (grades 1 or 2) was observed in the horses used within this study.

Although there is a greater incidence of tearing in the DL branch of the racehorses, this does not appear to be significantly contributing to an alteration in the mechanical properties of the ligament, with no differences in the structural and material properties of the DL branch between the two groups of horses being evident. Additionally, no differences in the mechanical properties of the DL were found to exist between the pathological and non-pathological specimens. Laxity of the dorsal aspect of the DL has been previously observed in a number of specimens with damaged ligaments (Whitton, McCarthy et al. 1997), and a study examining the mechanical properties of the CCL in dogs, found that laxity was greater in those dogs predisposed to CCL failure (Comerford 2002). Although laxity could not be quantified in this study due to the limitations of the

testing procedure, the biomechanical results suggest that neither racing nor a mild degree of ligament tearing are significantly altering the mechanical properties of the DL branch of the MPICL. However, the degree of tearing was only mild in the horses used within this study and hence this does not unequivocally prove that a relationship would not exist between more severe ligament tearing and a reduction in the mechanical properties of the ligament, as might be intuitively expected.

The collagen metabolism of the ligament is different between the two groups of horses. Within the DL branch of the racehorses the level of immature and mature cross-links was significantly increased compared to the non-raced, suggesting that elevated collagen remodelling is being induced in the DL branch of racehorses. Interestingly, there were no differences in the cross-link composition between the pathological and non-pathological samples. Additionally, the thermal properties of the DL branch were significantly altered in the two groups of horses. The T_{max} (the denaturation temperature of the collagen triple helix) was significantly greater in the racehorses, suggesting increased stability of the collagen triple helix in the racehorses. This again was not evident between the pathological and non-pathological specimens, thus suggesting that ligament tearing is not mediating an increase in collagen metabolism or conversely, that the increased collagen metabolism is not contributing to ligament pathology. This is also supported by the biochemical findings of the DM branch, in which there was an increased level of the immature and mature cross-link content, suggestive of increased collagen remodelling, in the raced compared to non-raced horses despite there being no differences in the degree of tearing.

Furthermore, relatively few relationships were found to exist between the mechanical and biochemical properties of the DL and DM branches of the MPICL in the raced and non-raced horses, which may indicate that while racing and race-training is causing an increase in collagen remodelling in the DL and DM branches, this may not be contributing to ligament failure but may be a physiological response of the DL and DM branches to increased exercise.

So why is tearing of the DL branch more prevalent in horses that have raced compared to those that have not raced if the major component of ligaments, collagen, is not

contributing to ligament failure and the mechanical properties of the ligament are not altered? It is known that during axial loading, the Cr impacts onto the radial facet of the C3 causing substantial palmar to dorsal and lateral to medial movement of the bone (Phillips and Wright 1994). The anatomical position of the DL branch and the direction of its fibres are biomechanically suited to resist the movements of the Cr relative to the C3 at the limit of carpal extension (Whitton, McCarthy et al. 1997), and a role of the MPICL to resist dorsal displacement of the Cr has been proposed (Whitton and Rose 1997). Tearing may therefore be the result of increased cyclical tensing of the ligament during racing and race-training, with complete rupture occurring when excessive forces are applied to the Cr causing dorsal displacement of the proximal row of the carpal bones. Furthermore tearing of the DL branch was not only limited to the racehorses, but a degree of tearing was also noted in the non-racehorses thus suggesting that even sub-maximal exercise can contribute to ligament tearing.

The material and structural properties were also found to be significantly different between the DM and DL branches, with the load required to fail the DM branch being greater. This suggests that the DM branch can withstand more stress than the DL, which may explain why it is the DL branch that is most commonly found to be ruptured upon arthroscopy (Phillips and Wright 1994). Furthermore, in an electron microscopic study of the intercarpal ligaments of the carpus by Davanaker *et al.*, (1996) they demonstrated that the DL branch had a larger number of smaller collagen fibrils compared to the DM, which they suggested may be a result of damage and degeneration of the ligament (Davankar, Deane et al. 1996). However, no reference was made to the integrity of the ligaments examined and these smaller fibrils may therefore be a normal structural feature of this branch (Firth, Deanne et al. 1991), suggesting further that this ligament is inherently more susceptible to failure.

There also appeared to be a relatively insignificant influence of age on the structural, material, or biochemical properties of the DL and DM branches, with age only significantly correlating with the tensile strength and linear stiffness of the DM branches. A previous histopathological study of the MPICL by Whitton *et al.*, (1999) found that degeneration was associated with increasing age, results which were not illustrated in this

study, suggesting further that rupture of the MPICL may be due to a single traumatic event rather than degenerative.

Summary

There is an increased prevalence of tearing of the DL branch of the MPICL with racing. This tearing, without an alteration in the mechanical properties of the DL branch of the MPICL and the anatomical structure and positioning of this branch may suggest that tearing and rupture of the MPICL is of little clinical significance, and that tearing observed during arthroscopy of horses that present with signs of midcarpal joint disease may be an independent finding rather than a cause of lameness.

Although the relative proportion of which this tearing contributes to the instability of the midcarpal joint and hence causes osteochondral damage cannot be elucidated from the results represented in this study, in the succeeding Chapter the relationship between DL and DM tearing and osteochondral damage, determined from articular cartilage damage, will be discussed.

CHAPTER FIVE

Cartilage Pathology and Its Relationship with the Inorganic and Organic Properties of Bone, and Ligament Pathology

5.1 CARTILAGE PATHOLOGY AND ITS RELATIONSHIP WITH THE INORGANIC AND ORGANIC PROPERTIES OF BONE

5.1.1 Introduction

Changes to both the subchondral bone and the articular cartilage are common features in joint disease, however, the initial event(s) that cause disease is subject to considerable debate. Studies have shown a relationship to exist between bone density and bone volume with articular cartilage damage in human and animal models of knee OA and osteoarthrosis (Wu, Burr et al. 1990; Carlson, Loeser et al. 1996; Matsui, Shimizu et al. 1997; Messner, Fahlgren et al. 2000; Bobinac, Spanjol et al. 2003; Pastoureau, Leduc et al. 2003). Studies have also documented biochemical changes in human OA bone tissues (Li and Aspden 1997; Mansell, Tarlton et al. 1997; Mansell and Bailey 1998). These studies showed that although OA bone tissue is sclerotic, it is undermineralised, and metabolically more active, with an increase in TGF-β, MMP-2 and BAP. Whether the increased metabolism of the subchondral bone is a consequence of or precedes cartilage erosion cannot be determined from these studies.

In the horse, cartilage fibrillation is often apparent in the articular cartilage overlying the most common sites of osteochondral fracture (Firth, Delahunt et al. 1999). A previous study on the Cr and C3 of treadmill exercised horses has shown an increase in bone sclerosis to be localised to those regions underlying common sites of cartilage degradation, however, the relationship between the degree of cartilage pathology and sclerosis was not determined (Firth, Delahunt et al. 1999). More recently, a magnetic resonance imaging (MRI) study by Anatastasiou *et al.*, (2003) of the distal row of equine carpal bones from Standardbred Trotters and Swedish Warmbloods with varying degrees of articular cartilage damage, found that cartilage lesions and cartilage loss were related to the sclerotic state of the underlying bone (Anastasiou, Skioldebrand et al. 2003).

Clearly a relationship between bone density and cartilage degradation exists in the carpus of the horse, but the relationship between cartilage fibrillation and the inorganic properties and the molecular composition of the Cr and C3 of clinically normal racing TBs has not been elucidated. The aim of this part of the study was to determine if these bone properties in the cortical bone (layer a) of the Cr and C3 were significantly altered in those horses (raced and non-raced) with a greater degree of cartilage pathology and/or in those that had raced. A relationship between racing and cartilage pathology with the inorganic and biochemical properties of bone was additionally investigated, to ascertain if those horses which had raced and had cartilage pathology had different bone density, morphometry measurements and/or biochemical properties. In Chapters Two and Three it was shown that age correlated with many of the markers of collagen metabolism and to a lesser extent the inorganic properties of Cr and C3, hence this factor was accounted for in the analysis.

5.1.2 Materials and Methods

5.1.2.1 Equine Samples and Cartilage Pathology Grading

Table 5.1 shows the age and gender of the horses used in this part of the study.

The scoring system used to grade the degree of cartilage pathology is that previously described in Chapter Two section 2.2.1.2.

Raced	Age	Gender	Non-raced	Age	Gender
R1	7	G	**1**	17	M
R2	7	M	**2**	NA	G
R3	8	G	**3**	15	G
R4	4	C	**4**	22	G
R5	6	C	**5**	9	M
R6	7	G	**6**	12	G
R7	7	C	**7**	10	M
R8	7	C	**8**	11	M
R9	6	C	**9**	8	M
R10	6	C	**10**	NA	G
R11	7	G	**11**	3	G
R12	4	G	**12**	6	G
R13	4	C	**13**	11	M
R14	4	F	**14**	17	G
			15	7	G
			16	17	G
			17	14	G

Table 5.1: The age (years) and gender the horses. (C= Colt, F= Filly G= Gelding (neutered male), M=mare, NA= Not Known).

5.1.2.2 Statistical Analysis

Statistical analysis was performed using SPSS v11.5 (SPSS Inc. USA) and Graphpad Prism v3.0 (Graphpad Software Inc. USA). Dr Gina Pinchbeck from the University of Liverpool aided in the analysis of the data.

Spearman's rank order correlation was performed to determine any influence of age on cartilage pathology. Significance was set at $P<0.05$ (both the ages of the raced and non-raced horses were included).

To evaluate the relationship between cartilage pathology or racing with the values of the various parameters quantified, while accounting for the confounding influence of age, linear regression models were used. The values for layer a (cortical layer) only were used. If the data were not normally distributed the data was log transformed. The parameter quantified (i.e. BMD, PICP) was the dependent variable, and age, racing status (raced or non-raced) and cartilage pathology score (raced and non-raced scores pooled) were the independent variables. Any interaction between racing and cartilage pathology score was also tested for all the parameters quantified. Where paired samples of left and right carpi were included, mixed effects linear regression models were used with horse identification as a random effect to allow for within horse clustering.

5.1.3 Results

5.1.3.1 Cartilage Pathology in the Raced and Non-raced Horses

The degree of cartilage pathology in the right and left Cr and C3 of the raced and non-raced horses used within this study are shown in Table 5.2. For the cartilage score of each individual cadaver see Appendix Two.

1) Cr:

a) Cartilage Grade 1+ versus Grade 0 (raced and non-raced pooled)

There were fewer carpi with grade 1 and above (1+) compared to those which had grade 0 in both the right and left; 12 out of 30 (40%) of the right Cr had a grade 1+ compared to 18 out of 30 (60%) with a grade 0. The values were the same in the left Cr.

b) Raced versus Non-raced Horses

Those carpi having grade 1+ did not vary greatly between the racehorses and non-racehorses; 6 out of 14 (42%) of the right Cr of racehorses had cartilage grading 1+, 6 out of 16 (37.5%) of the right Cr of non-racehorses had grade 1+, 5 out of 14 (35.7%) of the left Cr of racehorses had grade 1+ and 7 out of 16 (43.7%) from the non-racehorses had grade 1+.

2) C3:

a) Cartilage Grade 1+ versus Grade 0 (raced and non-raced pooled)

In contrast to the Cr, there were a greater number of carpi which had a cartilage grade 1+ compared to those which had grade 0 in both the right and left C3; 20 out of 30 (67%) of the right C3 had a grade 1+ compared to 10 out of 30 (33%) with a grade 0. The values were the same in the left C3.

b) Raced versus Non-raced Horses

Those carpi having grade 1+ was slightly greater in the racehorses compared to the non-racehorses, especially in the left C3; 8 out of 14 (57.1%) of the right C3 of racehorses had cartilage grading 1+, 8 out of 16 (50%) of the right Cr of non-racehorses had grade 1+, 11 out of 14 (78.6%) of the left Cr of racehorses had grade 1+ and 9 out of 16 (56.2%) from the non-racehorses had grade 1+.

Grade	Raced				Non-Raced			
	Right Cr	Left Cr	Right C3	Left C3	Right Cr	Left Cr	Right C3	Left C3
0	8 (57.1)	9 (56.3)	2 (14.3)	3 (21.4)	10 (62.5)	9 (56.3)	8 (50)	7 (43.8)
1	4 (28.6)	2 (14.3)	4 (28.6)	5 (35.7)	5 (31.3)	5 (31.3)	4 (25)	4 (25)
2	2 (14.3)	1 (7.1)	8 (57.1)	4 (28.6)	1 (6.3)	2 (12.5)	4 (25)	4 (25)
3	0 (0)	2 (14.3)	0 (0)	2 (14.3)	0 (0)	0 (0)	0 (0)	1 (6.3)
4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 5.2: The degree of cartilage pathology in the right and left Cr and C3 of the raced and non-raced horses (number of carpi and %).

5.1.3.2 The Relationship of Age with Cartilage Pathology

The degree of cartilage pathology did not significantly correlate with age in the right Cr ($p=0.67$, $r^2= -0.08$), the left Cr ($p=0.78$, $r^2= -0.05$), the right C3 ($p=0.32$, $r^2= -0.19$) nor the left C3 ($p=0.94$, $r^2= -0.01$).

5.1.3.3 The Relationships of Cartilage Pathology and Racing with the Inorganic and Organic Properties of the Cr and C3

Pathological samples refer to the articular cartilage of the Cr and C3 graded 1 or above (1+), and the non-pathological refer to those graded 0 (0). An effect of those horses with a pathology score of 1, 2 or 3 was investigated, however, no differences were observed, so to increase the power of the data, and to avoid including too many parameters within the models, those with a pathology score of 1 or above (1+) were pooled.

Unless stated, no interaction between racing and cartilage score was found, again this was left out of the final model to avoid including too many parameters within the models.

5.1.3.3.1 Cr:

a) BMD (Whole bone section and ROI) (g/cm^3)

Table 5.3 shows the outcome of the multivariable linear regression model of the BMD in layer a of the Cr. BMD (whole bone and ROI) was found to be significantly greater in those horses (raced and non-raced) with a cartilage score of grade 1+. In the whole bone section, BMD was significantly greater in those horses that had raced, regardless of the significant relationship with age. A negative interaction between racing and a pathology score of 1+ was also evident in the whole bone section p=0.02 (and not quite significant in the ROI (p=0.09)).

Variable	Coefficient (+/-Std. Error)	P Value
Whole Bone Section		
Cartilage score:		
0	Ref.	
1+	0.07+/-0.02	**0.02**
Racing status:		
Non-raced	Ref.	
Raced	0.05+/-0.02	**0.04**
Racing and score (1+) interaction:	-0.09+/-0.03	**0.02**
Age:	0.007+/-0.002	**0.02**
ROI		
Cartilage score:		
0	Ref.	
1+	0.14+/-0.06	**0.03**
Racing status:		
Non-raced	Ref.	
Raced	0.11+/-0.05	0.09
Racing and score (1+) interaction:	-0.14+/-0.07	0.09
Age:	0.01+/-0.006	**0.03**

Table 5.3: Multivariable linear regression model of the effects of cartilage pathology, racing status, age, and racing and score (1+) interaction on the BMD in layer a of the Cr (Ref.= reference category).

b) Histomorphometry

Bone perimeter (B.Pm) normalised against tissue area (T.Ar) (B.Pm./T.Ar mm^{-1}) ($p=0.007$) and bone perimeter (B.Pm) normalised against bone area (B.Ar) (B.Pm./B.Ar mm^{-1}) ($p=0.001$) (both expressed as mm^{-1}) were found to be significantly less in the racehorses. Bone perimeter (B.Pm./B.Ar mm^{-1}) was additionally significantly less in those horses that had a cartilage score of grade 1+ ($p=0.01$). An interaction between those horses that had raced and had a pathology score of 1+ was found to be not quite significant ($p=0.09$) for the bone perimeter (B.Pm./B.Ar mm^{-1}) (refer to Table 5.4).

Variable	Coefficient (+/-Std. Error)	P Value
Bone Area (B.Ar./T.Ar %)		
Cartilage score:		
0	Ref.	
1+	6.56+/-3.1	0.054
Racing status:		
Non-raced	Ref.	
Raced	2.83+/-3.45	0.42
Age:	0.15+/-0.46	0.75
Bone Perimeter (B.Pm./T.Ar mm^{-1})		
Cartilage score:		
0	Ref.	
1+	-0.25+/-0.22	0.27
Racing status:		
Non-raced	Ref.	
Raced	-0.77+/-0.24	**0.007**
Age:	0.03+/-0.03	0.29
Bone Perimeter (B.Pm./B.Ar mm^{-1})		
Cartilage score:		
0	Ref.	
1+	-0.62+/-0.22	**0.01**
Racing status:		
Non-raced	Ref.	
Raced	-1.009+/-0.25	**0.001**
Racing and score (1+) interaction:	0.73+/-0.41	0.09
Age:	0.32+/-0.34	0.34

Table 5.4: Multivariable linear regression model of the effects of cartilage pathology, racing status, age and, racing and score (1+) interaction on the histomorphometry in layer a of the Cr (Ref.= reference category).

c) Total Ca and Pi (% dry weight)

The total Ca and Pi content was not significantly greater in those horses that had a cartilage score of 1+, however, Ca and Pi of the racehorses was significantly higher (p=0.04) (refer to Table 5.5).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	-1.04+/-3.78	0.78
Racing status:		
Non-raced	Ref.	
Raced	9.69+/-4.43	**0.04**
Age:	1.05+/-0.51	0.06

Table 5.5: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the Ca and Pi content in layer a of the Cr (Ref.= reference category).

d) Total Immature and Mature Cross-link Content (mole/mole collagen)

There was no relationship found to exist with racing, cartilage pathology or age and the total immature and mature cross-link content (refer to Table 5.6).

Variable	Coefficient (+/-Std. Error)	P Value
Total Immature Cross-links		
Cartilage score:		
0	Ref.	
1+	0.06+/-0.11	0.57
Racing status:		
Non-raced	Ref.	
Raced	-0.14+/-0.15	0.35
Age:	-0.009+/-0.01	0.58
Total Mature Cross-links		
Cartilage score:		
0	Ref.	
1+	0.04+/-0.08	0.62
Racing status:		
Non-raced	Ref.	
Raced	-0.15+/-0.10	0.15
Age:	-0.003+/-0.01	0.76

Table 5.6: Multivariable linear regression model of the effects of cartilage pathology, racing status, and age the cross-link content in layer a of the Cr (Ref.= reference category).

e) Collagen Content (% dry weight)

The collagen content was found to have no relationship with the racing status, cartilage pathology or age (refer to Table 5.7).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	-0.07+/-0.06	0.27
Racing status:		
Non-raced	Ref.	
Raced	0.07+/-0.08	0.38
Age:	0.011+/-0.009	0.21

Table 5.7: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the collagen content in layer a of the Cr (Ref.= reference category).

f) Hydroxylysine Content (mole/mole collagen)

There was no relationship with cartilage pathology, racing or age and the hydroxylysine content (refer to Table 5.8).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	0.09+/-0.08	0.32
Racing status:		
Non-raced	Ref.	
Raced	0.17+/-0.13	0.21
Age:	0.004+/-0.01	0.78

Table 5.8: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the hydroxylysine content in layer a of the Cr (Ref.= reference category).

g) Collagen Type I Synthesis (PICP) (µg/l)

A significant relationship between the PICP content and age was evident (p=0.001), regardless of racing status and cartilage pathology (refer to Table 5.9).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	0.05+/-0.06	0.41
Racing status:		
Non-raced	Ref.	
Raced	-0.11+/-0.08	0.16
Age:	-0.03+/-0.009	**0.001**

Table 5.9: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the PICP content in layer a of the Cr (Ref.= reference category).

h) BAP (units/mg dry weight)

Those horses (raced and non-raced) with a cartilage score of grade 1+ had significantly higher levels of BAP (p=0.04) compared to those with no cartilage pathology. The BAP of the raced racehorses was also significantly higher (p=0.002), regardless of the significant relationship with age (p=0.01) (refer to Table 5.10).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	29.1+/-12.8	**0.04**
Racing status:		
Non-raced	Ref.	
Raced	55+/-14.9	**0.002**
Age:	-4.9+/-1.7	**0.01**

Table 5.10: Multivariable linear regression model of the effects of cartilage pathology, racing status, and age on the levels of BAP in layer a of the Cr (Ref.= reference category).

i) MMP-2 and -9 Expression (% std)

There was no relationship with racing or cartilage pathology and the levels of MMP-2 and-9. However, a relationship with MMP-2 and –9 levels and age was evident (refer to Table 5.11).

Variable	Coefficient (+/-Std. Error)	P Value
MMP-2		
Cartilage score:		
0	Ref.	
1+	2.16+/-5.4	0.69
Racing status:		
Non-raced	Ref.	
Raced	-7.93+/-6.8	0.26
Age:	-2.66+/-0.7	**0.004**
MMP-9		
Cartilage score:		
0	Ref.	
1+	5.92+/-14.12	0.68
Racing status:		
Non-raced	Ref.	
Raced	-23.80+/-19.87	0.25
Age:	-7.82+/-2.26	**0.003**

Table 5.11: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the MMP-2 and –9 activity in layer a of the Cr (Ref.= reference category).

j) TIMP-2 Expression (% std)

The levels of TIMP-2 were found to have no relationship with racing, cartilage pathology or age (refer to Table 5.12).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	-1.42+/-4.32	0.74
Racing status:		
Non-raced	Ref.	
Raced	-10.81+/-8.68	0.25
Age:	-0.76+/-1.70	0.66

Table 5.12: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the TIMP-2 activity in layer a of the Cr (Ref.= reference category).

k) COL23/4C$_{short}$ Neoepitope Expression (µg/ml)

The COL23/4C$_{short}$ expression was found to have no relationship with cartilage pathology, racing or age (refer to Table 5.13).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	0.27+/-0.33	0.43
Racing status:		
Non-raced	Ref.	
Raced	0.52+/-0.39	0.22
Age:	0.04+/-0.3	0.28

Table 5.13: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the COL23/4C$_{short}$ expression in layer a of the Cr (Ref.= reference category).

l) DSC – Tmax and Enthalpy (whole peak)

The Tmax (°C) and enthalpy (J/mg (dry weight)) was found to have no relationship with the racing status, cartilage pathology or age (refer to Table 5.14).

Variable	Coefficient (+/-Std. Error)	P Value
Enthalpy		
Cartilage score:		
0	Ref.	
1+	-0.14+/-5.99	0.98
Racing status:		
Non-raced	Ref.	
Raced	-5.02+/-7.32	0.51
Age:	1.25+/-1.71	0.48
Tmax		
Cartilage score:		
0	Ref.	
1+	0.13+/-1.82	0.94
Racing status:		
Non-raced	Ref.	
Raced	-0.003+/-2.01	0.99
Age:	0.03+/-0.47	0.93

Table 5.14: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the enthalpy and Tmax in layer a of the Cr (Ref.= reference category).

5.1.3.3.2 C3:

a) BMD (Whole bone section and ROI) (g/cm^3)

Table 5.15 shows the outcome of the multivariable linear regression model of the BMD of layer a of the C3. BMD of the whole bone section was found to be significantly greater in those horses (raced and non-raced) with a cartilage score of grade 1+ (p=0.0009) and not quite significant in the ROI (p=0.07). BMD of the whole bone section was also significantly greater in the racehorses (p=0.003). However, those horses that had raced and had a pathology score of 1+ had the lowest BMD (p=0.003).

Variable	Coefficient (+/-Std. Error)	P Value
Whole Bone Section		
Cartilage score:		
0	Ref.	
1+	0.05+/-0.01	**0.0009**
Racing status:		
Non-raced	Ref.	
Raced	0.07+/-0.02	**0.003**
Racing and score (1+) interaction:	-0.07+/-0.02	**0.003**
Age:	0.002+/-0.001	0.111
ROI		
Cartilage score:		
0	Ref.	
1+	0.03+/-0.019	0.08
Racing status:		
Non-raced	Ref.	
Raced	0.02+/-0.02	0.27
Age:	-0.001+/-0.002	0.55

Table 5.15: Multivariable linear regression model of the effects of cartilage pathology, racing status, age and, racing and score (1+) interaction on the BMD in layer a of the C3 (Ref.= reference category).

b) Histomorphometry

Bone area (B.Ar) normalised against tissue area (T.Ar) and expressed as a percentage (B.Ar./T.Ar %), bone perimeter (B.Pm) normalised against tissue area (T.Ar) (B.Pm./T.Ar mm^{-1}) and bone perimeter (B.Pm) normalised against bone area (B.Ar)

(B.Pm./B.Ar mm^{-1}) (both expressed as mm^{-1}) were found to have no relationship with racing status, pathology score or age (refer to Table 5.16).

Variable	Coefficient (+/-Std. Error)	P Value
Bone Area (B.Ar./T.Ar %)		
Cartilage score:		
0	Ref.	
1+	0.63+/-1.37	0.65
Racing status:		
Non-raced	Ref.	
Raced	-0.75+/-1.45	0.61
Age:	-0.21+/-0.19	0.28
Bone Perimeter (B.Pm./T.Ar mm^{-1})		
Cartilage score:		
0	Ref.	
1+	-0.34+/-0.41	0.41
Racing status:		
Non-raced	Ref.	
Raced	0.71+/-0.43	0.12
Age:	0.006+/-0.05	0.90
Bone Perimeter (B.Pm./B.Ar mm^{-1})		
Cartilage score:		
0	Ref.	
1+	-0.29+/-0.51	0.57
Racing status:		
Non-raced	Ref.	
Raced	0.75+/-0.55	0.19
Age:	0.03+/-0.07	0.59

Table 5.16: Multivariable linear regression model of the effects of cartilage pathology, racing status, and age on the histomorphometry in layer a of the C3 (Ref.= reference category).

c) Total Ca and Pi (% dry weight)

The Ca and Pi content was not significantly greater in those horses that had a cartilage score of 1+, or in those that had raced. There was a significant relationship between age and the Ca and Pi content (p=0.03) (refer to Table 5.17).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	-0.32+/-2.30	0.88
Racing status:		
Non-raced	Ref.	
Raced	3.25+/-2.67	0.23
Age:	0.56+/-0.26	**0.03**

Table 5.17: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the Ca and Pi content in layer a of the C3 (Ref.= reference category).

d) Total Immature and Mature Cross-link Content (mole/mole collagen)

There was no relationship found to exist with racing, cartilage pathology or age and the total immature and mature cross-link content (refer to Table 5.18).

Variable	Coefficient (+/-Std. Error)	P Value
Total Immature Cross-links		
Cartilage score:		
0	Ref.	
1+	0.08+/-0.13	0.52
Racing status:		
Non-raced	Ref.	
Raced	-0.109+/-0.17	0.53
Age:	0.02+/-0.01	0.26
Total Mature Cross-links		
Cartilage score:		
0	Ref.	
1+	-0.024+/-0.10	0.81
Racing status:		
Non-raced	Ref.	
Raced	-0.05+/-0.12	0.66
Age:	0.02+/-0.01	0.08

Table 5.18: Multivariable linear regression model of the effects of cartilage pathology, racing status, and age on the cross-link content in layer a of the C3 (Ref.= reference category).

e) Collagen Content (% dry weight)

The collagen content was found to have no relationship with the racing status, cartilage pathology or age (refer to Table 5.19).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	1.36+/-9.01	0.88
Racing status:		
Non-raced	Ref.	
Raced	3.54+/-10.31	0.73
Age:	-0.36+/-1.04	0.73

Table 5.19: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the collagen content in layer a of the C3 (Ref.= reference category).

f) Hydroxylysine Content (mole/mole collagen)

There was no relationship with cartilage pathology or age and the hydroxylysine content. However, the hydroxylysine content was not quite significantly greater in the racehorses (p=0.08) (refer to Table 5.20).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	-0.10+/-0.12	0.40
Racing status:		
Non-raced	Ref.	
Raced	0.29+/-0.15	0.08
Age:	0.02+/-0.01	0.20

Table 5.20: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the hydroxylysine content in layer a of the C3 (Ref.= reference category).

g) Collagen Type I Synthesis (PICP) (µg/l)

A relationship with the PICP content and age was evident (p=0.004), regardless of racing status and cartilage pathology (refer to Table 5.21).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	-0.08+/-0.07	0.28
Racing status:		
Non-raced	Ref.	
Raced	0.06+/-0.08	0.47
Age:	-0.03+/-0.01	**0.004**

Table 5.21: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the PICP content in layer a of the C3 (Ref.= reference category).

h) BAP Content (units/mg dry weight)

BAP was found to be significantly greater in the racehorses (p=0.0003), regardless of the significant relationship with age (p=0.02). There was no significant relationship with cartilage pathology (refer to Table 5.22).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	0.005+/-0.10	0.95
Racing status:		
Non-raced	Ref.	
Raced	0.58+/-0.12	**0.0003**
Age:	-0.03+/-0.01	**0.02**

Table 5.22: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the BAP content in layer a of the C3 (Ref.= reference category).

i) MMP-2 and -9 Expression (% std)

There was no relationship with racing or cartilage pathology and the levels of MMP-2 and-9. However, a relationship with MMP-2 and –9 levels and age was evident (refer to Table 5.23).

Variable	Coefficient (+/-Std. Error)	P Value
MMP-2		
Cartilage score:		
0	Ref.	
1+	-0.03+/-0.27	0.91
Racing status:		
Non-raced	Ref.	
Raced	0.28+/-0.33	0.40
Age:	-0.07+/-0.03	**0.05**
MMP-9		
Cartilage score:		
0	Ref.	
1+	-13.22+/-23.68	0.58
Racing status:		
Non-raced	Ref.	
Raced	28.66+/-28.40	0.33
Age:	-6.39+/-2.91	**0.04**

Table 5.23: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the MMP-2 and –9 expression in layer a of the C3 (Ref.= reference category).

j) TIMP-2 Expression (% std)

The TIMP-2 levels were found to have no relationship with racing, cartilage pathology or age (refer to Table 5.24).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	0.06+/-0.13	0.62
Racing status:		
Non-raced	Ref.	
Raced	0.15+/-0.20	0.45
Age:	0.04+/-0.04	0.35

Table 5.24: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the TIMP-2 expression in layer a of the C3 (Ref.= reference category).

k) COL23/4C$_{short}$ Neoepitope Expression (µg/ml)

The COL23/4C$_{short}$ expression was found to have no relationship with cartilage pathology or age. However, there is a possible effect of racing (p=0.15) (refer to Table 5.25).

Variable	Coefficient (+/-Std. Error)	P Value
Cartilage score:		
0	Ref.	
1+	-0.09+/-0.18	0.62
Racing status:		
Non-raced	Ref.	
Raced	0.31+/-0.20	0.15
Age:	-0.01+/-0.02	0.60

Table 5.25: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the COL23/4C$_{short}$ expression in layer a of the C3 (Ref.= reference category).

l) DSC – Tmax and Enthalpy (whole peak)

The Tmax (°C) and enthalpy (J/mg (dry weight)) was found to have no relationship with the racing status, cartilage pathology or age (refer to Table 5.26).

Variable	Coefficient (+/-Std. Error)	P Value
Enthalpy		
Cartilage score:		
0	Ref.	
1+	19.07+/-11.80	0.15
Racing status:		
Non-raced	Ref.	
Raced	-18.70+/-10.97	0.13
Age:	-3.07+/-1.70	0.12
Tmax		
Cartilage score:		
0	Ref.	
1+	1.03+/-2.91	0.73
Racing status:		
Non-raced	Ref.	
Raced	-1.43+/-2.80	0.62
Age:	0.55+/-0.40	0.22

Table 5.26: Multivariable linear regression model of the effects of cartilage pathology, racing status and age on the enthalpy and Tmax in layer a of the C3 (Ref.= reference category).

	Cr (Layer a)			C3 (Layer a)		
	Relationship with Cartilage Pathology (1+)	**Relationship with Racing**	**Relationship with Cartilage Pathology and Racing**	**Relationship with Cartilage Pathology (1+)**	**Relationship with Racing**	**Relationship with Cartilage Pathology and Racing**
BMD Whole Bone:	Sig* (+)	Sig * (+)	Sig* (-)	Sig*** (+)	Sig** (+)	Sig** (-)
BMD ROI:	NS	Sig* (+)	NS	NS	NS	NS
Bone Area (B.Ar./T.Ar%):	NS	NS	NS	NS	NS	NS
Bone Perimeter (B.Pm/T.Ar mm^{-1}):	NS	Sig** (-)	NS	NS	NS	NS
Bone Perimeter (B.Pm/B.Ar mm^{-1}):	Sig** (-)	Sig*** (-)	NS	NS	NS	NS
Ca and Pi:	NS	NS	NS	NS	NS	NS
Immature Cross-links:	NS	NS	NS	NS	NS	NS
Mature Cross-links:	NS	NS	NS	NS	NS	NS
Collagen Content:	NS	NS	NS	NS	NS	NS
Hydroxylysine:	NS	NS	NS	NS	NS	NS
PICP:	NS	NS	NS	NS	NS	NS
BAP:	Sig* (+)	Sig*** (+)	NS	NS	Sig*** (+)	NS
MMP-2:	NS	NS	NS	NS	NS	NS
MMP-9:	NS	NS	NS	NS	NS	NS
TIMP-2:	NS	NS	NS	NS	NS	NS
COL23/4C_{short}:	NS	NS	NS	NS	NS	NS
Enthalpy:	NS	NS	NS	NS	NS	NS
Tmax:	NS	NS	NS	NS	NS	NS

Table 5.27: Summary of the relationship of racing and cartilage pathology with the inorganic and organic properties of the Cr and C3. Sig= Significant results= *($P<0.05$), **($P<0.01$), ***($P<0.001$). Positive linear regression= (+). Negative linear regression= (-).

5.1.4 Discussion

A relationship between cartilage pathology and bone density previously reported in the carpus of horses (Anastasiou, Skioldebrand et al. 2003) was found to exist in the Cr and C3 of the TB horses within this study. The BMD was significantly greater in those horses that had a cartilage pathology score of one and above compared to those graded zero, further supporting the link between subchondral bone and the health of the overlying cartilage as first postulated by Radin *et al* (Radin and Rose 1986).

Racing was additionally found to have a significant relationship with BMD in layer a of the Cr and C3. This is probably a normal adaptive response of bone to increase its strength and hence protect the joint from damage when exposed to high-intensity exercise. An interaction between racing and cartilage pathology with BMD was also demonstrated in this study. Interestingly, however, those racehorses that had a cartilage pathology score of zero had the highest BMD score, thus suggesting that it is within the non-racehorses that the greatest interaction between cartilage pathology and BMD is most evident. These results may suggest that within the two groups of horses, pathology is being manifested in two forms. In the non-racehorses the relationship between BMD and cartilage pathology may represent signs of early OA, whereas in the racehorses the increased BMD but lack of cartilage pathology may be a consequence of the high-intensity exercise in an attempt to protect the bone in the short term, which in the long term may lead to degeneration of the cartilage.

However, this does not explain the negative relationship found to exist between racing and cartilage pathology with BMD. One explanation could be the reliability of the scoring system utilised in this study. It has been previously shown that microscopic changes to the cartilage may occur before gross signs of cartilage fibrillation are evident (Arokoski, Kiviranta et al. 1993; Price, Till et al. 1999). In a study that examined the collagen metabolism of the cartilage from the horses used within this study, it was found that there was a significant positive correlation between the proMMP-2 activity in the cartilage, a marker of cartilage degradation, and the BMD of the underlying cortical bone (layer a) in the racehorses and in the those racehorses with cartilage pathology (grade 1+) (C. Williams 2003, unpublished data). This demonstrates a link between bone

changes and cartilage degradation in the racehorses. This is in contrast to the macroscopic results shown here and may therefore question the use of gross cartilage scoring systems in isolation.

A relationship between BAP, a marker of bone formation, and cartilage pathology, was also found to exist in the horses in this study, being significantly greater in those horses with a cartilage score of one and above. BAP, which is expressed by mature osteoblasts, has been previously shown to correlate with cartilage damage in the synovial fluid of lame horses (Fuller, Barr et al. 2001) and increased levels have been shown in the subchondral bone of human late-stage OA specimens (Mansell, Tarlton et al. 1997; Mansell and Bailey 1998). Although the horses in this study only had a mild degree of cartilage fibrillation, the increased BAP in these horses demonstrates the closeness of the relationship between cartilage degradation and osteoblast activity. This increased BAP activity was also evident in the racehorses compared to the non-racehorses, suggesting that the mechanical loading experienced by the racehorse may also be mediating this response in the bone.

Furthermore, a relationship with bone perimeter and cartilage pathology, and with racing were also found to exist, as has previously been documented in human bone (Matsui, Shimizu et al. 1997; Bobinac, Spanjol et al. 2003). This further suggests that in those horses with cartilage pathology and those that had raced, bone formation is stimulated and bone resorption inhibited.

However, no significant interaction between racing and cartilage pathology with the BAP levels and histomorphometric measurements was evident, which supports the suggestion that within the two groups of horses, pathology is being manifested in two forms. The bone changes evident in the racehorses compared to the non-raced may be a consequence of the high-intensity exercise in an attempt to protect the bone, which in the short term is not significantly contributing to cartilage degradation. However, continued loading may eventually lead to degeneration of the cartilage, due to the altered biomechanics of the bone.

Few relationships were found to exist between cartilage pathology and the collagen metabolism of bone. Previous studies have found that the collagenous matrix in OA subchondral bone is metabolically more active (Mansell and Bailey 1998). However, these studies were conducted on late-stage OA patients, and the lack of severely degraded cartilage specimens within this study, may explain why this association was not apparent.

In addition, few relationships were found to exist between racing and the biochemical properties of the bone. Within Chapter Three, the collagen metabolism was shown to be significantly altered within the racehorses, particularly with regards to the expression of MMP-2 and-9 in layer a of the C3. This may be explained by the statistical analysis utilised within this study. The regression models used involve the inclusion of various independent variables, which may result in an over-parameterisation of the model, thus possibly reducing the power of the data and hence resulting in the loss of a significant difference between the raced and non-raced horses. A further explanation for this lack of relationship between racing and collagen metabolism could be because the greatest significant differences between the two groups of horses was evident in the deeper, trabecular regions (layers b-d) of the bone comparable to the cortical layer. It was felt inappropriate to analyse these layers of the bone since it was the interaction between the region of bone directly underlying the articular cartilage (layer a) that was most important in this study.

In summary, although this study cannot directly corroborate whether bone changes precede cartilage changes or cartilage degradation results in bone changes, it does further contribute to the understanding of the relationship known to exist between the articular cartilage and subchondral bone.

5.2 CARTILAGE PATHOLOGY AND ITS RELATIONSHIP WITH LIGAMENT PATHOLOGY

5.2.1 Introduction

The extent to which tearing of the intercarpal ligaments contributes to osteochondral damage, and specifically articular cartilage degeneration, in the carpal joint of the horse has been subject to debate for many years (McIlwraith 1992; Kannegieter and Colgan 1993; Phillips and Wright 1994; Whitton, Kannegieter et al. 1997; Whitton and Rose 1997). Studies in other species have found that intra-articular ligament damage causes degenerative joint disease, and experimental transection of the CCL (ACL) in the dog has long been used as a model for inducing stifle (knee) OA (Brandt, Myers et al. 1991). In the horse, a study by Kannegieter *et al.*, (1993) on the arthroscopic findings of 76 horses with carpal joint disease, found that joints with the most severe cartilage degradation also had a higher incidence of ligament lesions and it was suggested by the authors that such evidence was consistent with ligament damage leading to destabilisation of the joint and damage to the articulating cartilage (Kannegieter and Colgan 1993). However, further studies on horses with carpal joint disease have found no association to exist between ligament tearing and cartilage degeneration (McIlwraith 1992; Phillips and Wright 1994; Whitton, Kannegieter et al. 1997). These studies were primarily conducted on horses with either clinical or radiological signs of pathology in the midcarpal joint and to date no information exists on the relationships between ligament tearing and articular cartilage degeneration in clinically normal horses.

The aim of this part of the study was to ascertain whether any association exists between the degree of articular cartilage pathology of the Cr and C3 and the degree of ligament pathology of the DL and DM branches of the MPICL from horses with no clinical signs of orthopaedic disease that had been subject to racing and race-training prior to euthanasia.

5.2.2 Materials and Methods

5.2.2.1 Equine Samples and Ligament and Cartilage Pathology Grading

Table 5.28 shows the age and gender of the horses used in this part of the study.

The scoring systems used to grade the degree of ligament tearing and cartilage pathology are those previously described in Chapter Four 4.2.1.2 section and Chapter Two section 2.2.1.2 respectively.

Raced	Age	Gender	Non-raced	Age	Gender
R7	7	C	**2**	NA	G
R8	7	C	**3**	15	G
R9	6	C	**4**	22	G
R10	6	C	**5**	9	M
R11	7	G	**6**	12	G
R12	4	G	**7**	10	M
R13	4	C	**8**	11	M
R14	4	F	**9**	8	M
R15	5	G	**10**	NA	G
R16	9	G	**11**	3	G
R17	12	G	**12**	6	G
R18	8	G	**13**	11	M
R19	3	G	**14**	17	G
R20	4	F	**15**	7	G
R21	4	G	**16**	17	G
R22	3	C	**17**	14	G
R23	7	G	**18**	9	G
R24	2	C	**19**	6	G
			20	4	G
			21	13	M
			22	8	G
			23	12	G
			24	10	G

Table 5.28: The age (years) and gender the horses. (C= Colt, F= Filly G= Gelding (neutered male), M=mare, NA= Not Known).

5.2.2.2 Statistical Analysis

Spearman's rank order correlation was performed between the various observations. Significance was set at $P<0.05$.

5.2.3 Results

5.2.3.1 Ligament and Cartilage Pathology in the Raced and Non-raced Horses

The degree of ligament and cartilage pathology in the right and left DL, DM, Cr and C3 of the raced and non-raced horses used within this study are shown in Table 5.29a and b. For the ligament and cartilage score of each individual cadaver see Appendix Two.

Ligament Pathology:

The degree of ligament tearing in both the raced and non-raced horses was more prevalent in the DL branch of the MPICL compared to the DM branch; 52.5% (42 out of 80) of the carpal joints (right and left, raced and non-raced pooled) had grade 1 or above ligament pathology in the DL branch compared to 8.8% (7 out of 80) in the DM branch.

Ligament pathology in the right and left DL branch was slightly greater in the raced compared to the non-raced carpi; 76% (13 out of 17) of the right DL from the racehorses had a pathology grade 1+, compared to 50% (11 out of 22) of the right DL from the non-racehorses. Additionally, 61% (11 out of 18) of the left DL from the racehorses had a pathology grade 1+, compared to 30% (7 out of 23) of the left DL from the non-racehorses. This was not evident in the DM branch.

Cartilage Pathology:

The degree of cartilage pathology (grade 1+) in the right and left Cr of the racehorses compared to the non-racehorses was relatively similar, although pathology was slightly higher in the right racehorses; 41% (7 out of 17) of the right Cr from the racehorses had grade 1+, 27% (6 out of 22) of the right Cr from non-racehorses had grade 1+, 33% (6 out of 18) of the left Cr from racehorses had grade 1+ and 43% (10 out of 23) from the non-racehorses had grade 1+.

However, within the C3 those carpi having grade 1+ was greater in the racehorses compared to the non-racehorses; 71% (12 out of 17) of the right C3 from the racehorses had grade 1+ compared to 45% (10 out of 22) of non-racehorses. Additionally, 71% (14 out of 18) of the left C3 from racehorses had grade 1+ compared to 56% (13 out of 23) of the non-racehorses.

a) Ligament Pathology

Grade	Raced				Non-Raced			
	Right DL	Left DL	Right DM	Left DM	Right DL	Left DL	Right DM	Left DM
0	4 (24)	7 (39)	16 (94)	18 (100)	11 (50)	16 (70)	19 (86)	20 (87)
1	4 (24)	3 (17)	1 (6)	0 (0)	4 (18)	2 (9)	3 (14)	2 (9)
2	6 (35)	4 (22)	0 (0)	0 (0)	2 (9)	1 (4)	0 (0)	1 (4)
3	3 (18)	3 (17)	0 (0)	0 (0)	4 (18)	3 (13)	0 (0)	0 (0)
4	0 (0)	1 (6)	0 (0)	0 (0)	1 (5)	1 (4)	0 (0)	0 (0)

b) Cartilage Pathology

Grade	Raced				Non-Raced			
	Right Cr	Left Cr	Right C3	Left C3	Right Cr	Left Cr	Right C3	Left C3
0	10 (59)	12 (67)	5 (29)	4 (22)	16 (63)	13 (57)	12 (55)	10 (43)
1	7 (41)	5 (28)	2 (12)	5 (28)	6 (27)	8 (35)	6 (27)	6 (26)
2	0 (0)	0 (0)	8 (47)	6 (33)	0 (0)	2 (9)	4 (18)	5 (22)
3	0 (0)	1 (6)	1 (6)	2 (11)	0 (0)	0 (0)	0 (0)	2 (9)
4	0 (0)	0 (0)	1 (6)	1 (6)	0 (0)	0 (0)	0 (0)	0 (0)

Table 5.29: The degree pathology in the right and left a) DL and DM branches of the MPICL and b) Cr and C3 of the raced and non-raced horses (number of carpi and %).

5.2.3.2 The Relationships of Age with Ligament and Cartilage Pathology

The ages of the horses varied from 2 to 22 years (the ages of the raced and non-raced horses were pooled).

The degree of ligament tearing significantly positively correlated with age in the left DM (r^2= 0.43, p= 0.005), although there was no significant covariation with age (p=0.28). There was no correlation in the right DL (r^2= -0.21, p= 0.22), the left DL (r^2= 0.01, p= 0.93), or the right DM (r^2= 0.28, p= 0.08).

The degree of cartilage pathology did not significantly correlate with age in either the right (r^2= 0.08, p= 0.62) and left (r^2= 0.28, p= 0.08) Cr or the right (r^2= -0.03, p= 0.85) and left (r^2= 0.15, p= 0.33) C3.

5.2.3.3 Ligament and Cartilage Pathology Relationships (Correlations)

Table 5.30 shows the correlation coefficients of ligament and cartilage pathology grading in the raced and non-raced horses.

The degree of cartilage pathology was found to correlate negatively with the degree of ligament pathology in the raced, and in the raced and non-raced (data pooled) horses. The degree of cartilage pathology declined in the right and left Cr and C3 (data pooled), as the ligament pathology in the right and left DL and DM increased (data pooled).

	Raced		Non-raced		Raced & Non-raced	
	Right Cr & C3	**Left Cr & C3**	**Right Cr & C3**	**Left Cr & C3**	**Right Cr & C3**	**Left Cr & C3**
Right DL:	0.19	-	0.06	-	0.03	-
Left DL:	-	0.20	-	0.11	-	0.11
Right DM:	-0.09	-	0.07	-	-0.02	-
Left DM:	-	Too few with pathology	-	0.11	-	0.057
Right DL & DM:	-0.45**	-	-0.15	-	-0.24*	-
Left DL & DM:	-	-0.24	-	0.037	-	-0.09

Table 5.30: Correlation coefficients (Spearman r) of the ligament and cartilage pathology grading in the raced and non-raced horses. Significant results; *($P<0.05$), **($P<0.01$).

5.2.4 Discussion

The degree of tearing/pathology of the DL branch of the MPICL was found to be most prevalent in the raced compared to the non-raced horses. However, this difference between the two groups of horses was not apparent in the extent of articular cartilage degeneration of the Cr and C3, suggesting that although racing is contributing to ligament tearing, it does not contribute to an increased prevalence of gross cartilage pathology. Interestingly, an inverse relationship was shown between tearing of the right and left DL and DM branches and cartilage pathology in the right and left Cr and C3 of the racehorses. This is in contrast to other studies, which have either shown no association between MPICL tearing and cartilage degeneration (McIlwraith 1992; Phillips and Wright 1994; Whitton, Kannegieter et al. 1997) or a positive relationship (Kannegieter and Colgan 1993). However, unlike the horses in those studies, the horses in this study did not show any clinical signs of orthopaedic disease prior to euthanasia.

Whitton *et al.*, (1997) have shown an inverse relationship with MPICL tearing and subchondral bone damage (particularly with fracture score). The results from that study and from this study suggest that tearing of the MPICL does not significantly contribute to joint instability and the associated degeneration of the osteochondral unit, particularly in the short term, but that the tearing observed during arthroscopy of horses that present with signs of midcarpal joint disease may be an independent injury.

These results do not however unequivocally indicate that MPICL rupture may not contribute to some degree of osteochondral damage. Firstly, the rate of degeneration in CCL deficient dogs is very slow, despite it being the major contributor to restraint of cranial drawer, as shown in the human knee (Brandt, Myers et al. 1991). The MPICL of the horse is probably relatively less important in overall joint function than the CCL in dogs (Bramlage, Schneider et al. 1988), so it follows that degeneration of the cartilage resulting from MPICL tears might proceed extremely slowly (Whitton, Kannegieter et al. 1997), especially in the horses within this study which did not have severe ligament tearing (i.e. grades 3 or 4). Secondly, as outlined in section 5.1.4, gross articular cartilage scoring does not identify those changes to the cartilage that may be occurring at a microscopic/cellular level and hence may not truly reflect the degeneration occurring

within the cartilage. Finally, MPICL tearing will place increased stress on the other intercarpal ligaments, such as the DMICL (Whitton, Kannegieter et al. 1997) and desmitis of the intercarpal ligaments has been proposed as a major cause of lameness (Bramlage, Schneider et al. 1988).

In summary, the results from this study demonstrate that racing and race-training is contributing to some degree of ligament tearing, but this mild degree of tearing does not appear to be significantly contributing to pathology and hence gross signs of cartilage degeneration.

CHAPTER SIX

Discussion

6.1 INTRODUCTION

This thesis aimed to assess the response of the Cr and C3, and the MPICL of the midcarpal joint to racing and race-training and to gain an understanding of how this may be related to carpal diseases common in racing TBs. In order to ascertain this information, the mineral component and the ECM metabolism (synthesis and degradation) of the cortical and trabecular bone of the Cr and C3, and the biomechanics and the ECM metabolism of the MPICL was quantified on TB horses that had either been actively race-trained and raced up to the time of euthanasia, or on horses that had not been in race-training or raced.

In addition, the relationships between the inorganic and/or the organic matrix of the bone, and MPICL tearing, with articular cartilage degeneration, a sign of OA, in the raced and non-raced horses was determined.

The ages of the horses varied considerably from 3 to 22 years, thus representing a population of maturing, mature and ageing horses. Any relationships with age and the various parameters quantified were therefore investigated.

The principal findings, and possible consequences of these findings to the integrity of the midcarpal joint in the racing TB are discussed.

6.2 THE EFFECTS OF RACING ON THE MINERAL COMPONENT AND THE ECM METABOLISM OF THE Cr AND C3

Previous studies have assessed the effects of exercise on bone quantity and quality by obtaining measurements on the mineral component of the Cr and C3 via DEXA and histomorphometry (Young, Richardson et al. 1991; Firth, Delahunt et al. 1999) but these have primarily been obtained on TB horses subjected to a treadmill-exercise regime, which may not truly reflect the effect of racing and race-training on the skeleton. In addition, no studies to date exist on the effect of exercise on the ECM metabolism of the Cr and C3 of racehorses. This is the first study to assess these components of the midcarpal bones of racing TBs.

Within Chapter Two it was shown that racing and race-training was inducing osseous bone formation in both the cortical and trabecular regions of the Cr and C3. This is probably a normal adaptive response of bone to increase its strength and hence protect the joint from damage when exposed to high-intensity exercise. Interestingly, the volumetric bone density, being greatest in the cortical layer, did not decline as notably from layers a to d in the racehorses than it did in the non-racehorses, which was further reflected in the Ca and Pi and histomorphometrical data. This suggests that a net increase in bone formation is occurring within both the cortical and trabecular regions of the racehorse bone compared to the bone from those horses that had not raced.

In Chapter Three, it was shown that racing was causing elevated bone collagen remodelling, above the normal physiological response to exercise, in both the cortical and trabecular regions of the Cr and C3, which appears to result in a modified collagenous matrix. The ratio of immature to mature cross-links was significantly greater, and the HL-Pyr and Lys-Pyr content significantly less, in the racehorses compared to the non-racehorses, signifying an elevated collagen turnover, which may result in a less resilient bone. The level of lysine hydroxylation was also found to be greatest in the racehorse bone compared to the non-raced. This post-translational modification of the collagen fibrils may be an attempt at a repair mechanism, to combat the possible accumulation of microfractures resulting from the increased mechanical load during high-intensity exercise. Increased lysine hydroxylation may also result in the formation of thin collagen fibrils and the formation of fewer pyrrole cross-links (the

cross-link suggested to contribute most to the mechanical strength of the bone) and hence reduce the tensile strength of the bone.

Markers of collagen degradation, MMP-2 and -9 and the percentage cleaved collagen were also found to be elevated in the raced Cr and C3, although there were no differences in the TIMP-2 expression. This lack of TIMP-2 expression, despite the up-regulation of MMP-2, indicates that there is an imbalance of MMP and TIMP activity with high-intensity exercise, resulting in the increased degradation of the collagen molecules. This increase in collagen degradation may result in a mechanically weaker tissue.

In addition, the increased levels of the proteolytic enzymes may be contributing to the significantly lower enthalpy of denaturation, whereby the increased quantity of denatured and damaged collagen molecules will result in a disruption in the hydrogen bonding holding the triple helix together, hence reducing the energy required to reduce the native collagen triple helix to random coil i.e. the enthalpy of denaturation.

Interestingly it was predominantly within the trabecular regions of the bone, layers c and d, that the greatest significant differences in the levels of bone collagen synthesis, degradation and alterations in the thermal properties of the collagen between the two groups of horses were observed. This presumably reflects the higher turnover rate and hence the more immature state of the trabecular bone in these racehorses.

Taken together, the results from Chapters Two and Three suggest that while there is a net increase in bone formation in the racehorses, reflected in the BAP, PICP, histomorphometrical and BMD data and the increased Ca and Pi deposition throughout the depth of the bone, there is additionally an increase in bone collagen remodelling, particularly within the most trabecular regions of the bone. The increase in bone density would presumably result in a concomitant increase in the stiffness of the bone, and since the greatest bone density was within the cortical bone rather than the trabecular regions it may follow that within the Cr and C3 of racehorses failure of this 'stiffer' cortical bone may eventually result from its lack of support from a metabolically more active and hence less resilient trabecular bone.

6.3 CARTILAGE PATHOLOGY AND ITS RELATIONSHIP WITH THE INORGANIC AND ORGANIC PROPERTIES OF BONE

Changes to both the subchondral bone and the articular cartilage are common features in joint disease and a relationship between bone density and cartilage degradation has been demonstrated in the carpus of the horse (Firth, Delahunt et al. 1999; Anastasiou, Skioldebrand et al. 2003). However, the relationship between gross signs of cartilage pathology with racing and the inorganic and organic properties of the bone are unknown.

Chapter Five demonstrated that there was a relationship between bone density, bone perimeter and BAP levels (in the cortical layer (layer a) of the Cr and C3 of pooled raced and non-raced data) with the degree of cartilage pathology. The values of these parameters were greatest in those horses with a cartilage pathology score of one and above, further supporting the link between the health of the subchondral bone and the health of the overlying cartilage.

In addition, these parameters were found to have a positive relationship with racing. Interestingly however, there was a lack of interaction between racing and cartilage pathology with these parameters, possibly suggesting that it is within the non-racehorses that the greatest relationship between cartilage pathology and bone density, bone perimeter and BAP is most evident. These results may suggest that within the two groups of horses pathology is being manifested in two forms. The positive interaction between cartilage pathology and bone changes in the non-racehorses may represent signs of early OA. In the racehorses the presence of bone changes but lack of interaction with cartilage pathology may be a consequence of the high-intensity exercise in an attempt to protect the bone, which in the short term is not significantly contributing to cartilage degradation. However, it can not be excluded that continued loading may not eventually lead to degeneration of the cartilage due to the presumed altered biomechanics of the bone.

6.4 THE EFFECTS OF RACING ON THE MPICL

Biomechanics and Collagen Metabolism of the MPICL

The cause of MPICL injury and tearing is poorly understood, and the relationship between ligament tearing and high intensity exercise is unclear. Within Chapter Four the degree of ligament tearing, the biomechanics and collagen metabolism of the DL and DM branches of the MPICL from raced and non-raced horses was ascertained to address these queries. In addition, the relative contribution of an increased collagen metabolism to failure/ rupture of the ligament was explored.

Racing and race-training did cause a greater incidence of tearing/ pathology in the DL branch of the MPICL, although this was not apparent in the DM branch. In addition, racing did not appear to be significantly contributing to an alteration in the mechanical properties of the DL branch. Furthermore, no differences in the mechanical properties between pathological and non-pathological ligaments were observed, suggesting that the mild degree of ligament tearing apparent in the racehorses in this study was not significantly contributing to a reduction in the mechanical properties of the ligament.

There was however, a greater ratio of immature to mature cross-links in both the DL and DM branches of the MPICL of racehorses suggesting that the increased loading experienced by the midcarpal ligaments during racing was causing elevated collagen remodelling. Interestingly, it was apparent from these data that this did not appear to associate with ligament tearing.

So why is tearing of the DL branch more prevalent in horses that have raced compared to those that have not raced if the major component of ligaments, collagen, is not contributing to ligament failure and the mechanical properties of the ligament are not altered? It is possible that the greater prevalence of tearing is the result of increased cyclical tensing of the DL branch during racing and race-training, which is probably enhanced due to the direction of its fibres and anatomical positioning within the midcarpal joint.

Tearing of the MPICL and its Association with Osteochondral Damage

Tearing of the MPICL is commonly identified in horses presenting with clinical signs of carpal joint disease (McIlwraith 1992; Kannegieter and Colgan 1993; Phillips and Wright 1994), although a high prevalence of tearing has been observed in horses with no history of disease (Whitton, Kannegieter et al. 1997; Whitton and Rose 1997). The association between MPICL tearing and joint disease is therefore unclear. Correlation coefficients between ligament and cartilage pathology scores in the raced and non-raced horses, suggested that the increased tearing of the DL branch of the MPICL observed in the racehorses within this study did not significantly contribute to pathology and the associated degeneration of the cartilage in the short term.

Tearing of the DL branch of the MPICL observed during arthroscopy of horses that present with other signs of midcarpal joint disease may therefore be an independent finding.

6.5 RELATIONSHIPS WITH AGE

Relationships with age and the markers of collagen metabolism and to a lesser extent the inorganic properties of the Cr and C3 were documented in this study. These findings were similar to that previously reported in the horse and in humans. Bone porosity increased with age, signifying an increase in bone resorption with age, which has been documented in the navicular bone of horses (Gabrie, Detilleux et al. 1999). Markers of bone collagen synthesis and degradation generally declined with age, reflecting a reduction in the level of modelling and remodelling with maturation and growth. The thermal characteristics of the collagen, although more varied and complex, were found to increase with age suggesting a stabilisation of the triple helix with age. These findings reflect an age related 'slowing down' of collagen turnover.

Interestingly, few relationships with age and the structural or material or biochemical properties of the DL and DM branches of the MPICL were documented, which may suggest that tearing or rupture of the MPICL is not a product of degeneration with age.

6.6 OTHER FINDINGS

Right and Left Differences

Within the chapters the average data for the right and left carpi were presented since that represented the value of the individual horse. A paired t-test was performed between the left and right values to ascertain any differences between the left and right.

Few significant differences were found to exist between right and left, suggesting an even distribution of load between the right and left Cr and C3. Interestingly, any differences found were more frequent in the non-racehorses compared to the racehorses. This was thought to be either a chance finding, or may suggest that some horses preferentially lead with one forelimb more than the other or reflect the exercise undertaken by these 'pleasure' horses, most probably ménage schooling whereby horses are exercised in small arenas.

Cr versus C3

Significant differences in the parameters quantified between the racehorses and non-racehorses were apparent in both the Cr and C3, demonstrating the greater degree of loading experienced by the racing TB in the bones of the midcarpal joint compared to horses undergoing exercise of lower intensity. Interestingly however, the greatest significant differences observed appeared to be within the C3 and may reflect the increased load received by the C3 during racing and race-training, which in the long-term may have consequences for the integrity of this midcarpal bone.

Limitation of the Study

One of the limitations of this study is that the full exercise history of the non-racehorses was not known. All of the racehorses were in training, and would therefore be undergoing a more intensive exercise regime than the non-racehorses. However, the non-racehorses comprised a group of competition and 'pleasure' horses, to which it is likely a wide range of exercise histories applied. The non-racehorses cannot be regarded as perfect controls, but it can be safely assumed that they were undergoing distinctly lower intensity exercise than the racehorses.

Nevertheless, significant differences between the composition of raced and non-raced bone, cartilage and ligament, have been shown in this study, so had the non-raced group been more homogeneous controls limited to slow work only, it would be expected that the differences seen may have been even greater.

This study therefore specifically distinguishes between exercise and racing, and demonstrates the extent to which carpal joint disease is an occupational hazard of racing.

6.7 CONCLUSIONS

- Racing and race-training results in increased bone density, and Ca and Pi deposition in both the cortical and trabecular regions of the Cr and C3, possibly in an attempt to strengthen the bone.

- Racing and race-training causes elevated bone collagen remodelling, which appears to result in a modified collagenous matrix, particularly within the trabecular regions of the bone.

- Failure of a denser and presumably 'stiffer' cortical bone that is being supported by a metabolically more active trabecular bone may result with continued racing and race-training.

- The relationship known to exist between articular cartilage degradation and subchondral bone changes has been supported by the data presented here, particularly within the non-racehorses. The lack of interaction of bone changes with cartilage pathology in the racehorses may be a consequence of the high-intensity exercise in an attempt to protect the bone, which in the short term is not significantly contributing to cartilage degradation.

- Racing and race-training results in an increased prevalence of tearing of the DL branch of the MPICL. This does not appear to be mediated or contributing to an increased ECM metabolism, suggesting tearing is due to single traumatic event.

- Tearing and rupture of the MPICL does not contribute to pathology and hence gross cartilage degradation in the short term. Tearing would seem to be of little clinical significance and is an independent finding rather than a cause of lameness. In addition, tearing or rupture of the MPICL is not a cause of degeneration with age.

- Age correlates with many of the markers of collagen metabolism and to a lesser extent the inorganic properties of the Cr and C3, reflecting a reduction in the level of modelling and remodelling with maturation and growth.

6.8 FUTURE WORK

During the work undertaken for this thesis many results and observations were made, some of which could not be pursued in depth. Below are a number of areas that warrant further investigation.

Bone:

To investigate the mechanical properties of the bone

It has been suggested previously and in this thesis, that an increase in bone density would result in a concomitant increase in bone stiffness, which in the long-term may alter the share of the compressive load absorbed by the bone and cartilage, possibly having deleterious results. Quantification of the biomechanical properties of raced and non-raced bone would therefore provide valuable information as to the mechanical integrity of the bone.

To investigate the presence of the homotrimer in raced and non-raced bone

Type I collagen is a heterotrimer composed of two α1 chains and one α2 chains, in contrast to the other two major fibrous collagens, types II and III, both of which are homotrimers. The type I heterotrimer has presumably been conserved during evolution because of its greater stability, particularly its mechanical properties, since it occurs predominantly in tissues that are subject to increased mechanical loads. Additionally, homotrimer fibrils have been reported to be thinner, possess a reduced mechanical strength and reduced mineralisation of the bone (McBride, Choe et al. 1997; Misof, Landis et al. 1997). Recently the presence of the homotrimer has been documented in the subchondral bone of OA femurs (Bailey, Sims et al. 2002), in addition to an increased lysyl hydroxylation, increased diHLNL to HLNL ratio, increased Hyl-Pyr to Lys-Pyr ratio, and decreased enthalpy, which together have the potential to have a significant effect on the physical and biomechanical properties of the bone matrix.

The increased level of lysine hydroxylation, modified cross-link composition and lower enthalpy of the fibres in the racehorses within this study, findings similar to that reported by Bailey *et al.*, (2002), are suggestive that type I homotrimer is present in the bone of racehorses and hence warrant its investigation.

To investigate the presence of the pyrrole cross-link

Increased lysine hydroxylation is known to result in fewer pyrrole cross-links, the cross-link that contributes more to the tensile strength of bone than the pyridinoline cross-links (Knott, Whitehead et al. 1995). The increased lysine hydroxylation within the bone from racehorses may therefore result in fewer pyrrole cross-links and hence reduce the strength of the bone. Quantification of the pyrrole content is therefore warranted.

Ligament:

To further develop the ligament biomechanical testing protocol

The ligament biomechanical testing protocol employed in this thesis did not directly mimic the physiological conditions experienced *in vivo*. More information regarding the mechanical properties of the ligament could be obtained if *in vivo* conditions could be defined and an appropriate testing apparatus designed to replicate these conditions.

To investigate the presence of non-collagenous ECM proteins

Although 80% of the dry weight of ligaments is collagen, ligaments are also composed of non-collagenous ECM proteins, including PGs, which confer the viscoelastic properties of the ligament and hence together with collagen determine the mechanical properties of the ligament. Within this thesis the collagenous matrix did not appear to significantly contribute to the mechanical integrity of the ligament therefore study of the non-collagenous ECM proteins is warranted.

APPENDICES

APPENDIX ONE

List of Abbreviations

ACL	anterior cruciate ligament
ACP	adol condensation product
ADAM	a disintegrin and a metalloproteinase domain
ALP	alkaline phosphatase
ANOVA	analysis of variance
APMA	aminophenyl mercuric acetate
APS	ammonium persulphate
BAP	bone-specific alkaline phosphatase
B.Ar.	bone area
BMC	bone mineral content
BMD	bone mineral density
B.Pm.	bone perimeter
Ca	calcium
$CaCl_2$	calcium chloride
CCL	cranial cruciate ligament
COMP	cartilage oligomeric matrix protein
Cr	radial carpal bone
C2	second carpal bone
C3	third carpal bone
CTB	central tarsal bone
DDFT	deep digital flexor tendon
deH-HHL	dehydro-histidinohydroxylysinonorleucine
deH-HLNL	dehydro-hydroxylysinonorleucine
deH-LNL	dehydro-lysinonorleucine
DH	Dunkin-Hartley guinea pigs
dH_2O	deionised water
diHLNL	dihydroxy-lysinonorleucine
DL	dorsolateral

DM	dorsomedial
DMICL	dorsomedial intercarpal ligament
DSC	differential scanning calorimetry
ECM	extracelluar matrix
EDTA	ethylene diaminetetraacetic acid
ELISA	enzyme linked immunoabsorbant sandwich assay
GAG	glycosaminoglycans
Gly	glycine
HAc	acetic acid
HHL	histidinohydroxylysinonorleucine
HL-Pyr	hydroxylysylpyridinoline
HLNL	hydroxylysinonorleucine
HLKNL	hydroxylysino-5-keto-norleucine
HLONL	hydroxylysino-5-oxonorleucine
Hylys	hydroxylation of lysine
IGF	insulin-like growth factor
IL-1β	interleukin-1β
IL-4	interleukin-4
IL-6	interleukin-6
kg	kilogram
Lys-Pyr	lysylpyridinoline
LNL	lysinonorleucine
LONL	lysino-5-oxonorleucine
LPICL	lateral palmar intercarpal ligament
MCB	metacarpal bone
MCIII	third metacarpal bone
MCL	medial collateral ligament
MCP	metacarpophalangeal joint
mg	milligram
mm	millimetre
MMP	matrix metalloproteinase
MPICL	medial palmar intercarpal ligament

MTIII	third metatarsal bone
MT-MMP	membrane type-matrix metalloproteinase
MTLT	mineralising turkey leg tendon
OA	osteoarthritis
OC	osteochondrosis
OF	osteochondral fragmentation
OI	osteogenesis imperfecta
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PG	proteoglycan
Pi	inorganic phosphate
PICP	procollagen type I carboxy terminal propeptide
PL	palmarolateral
PM	palmaromedial
RER	rough endoplasmic reticulum
RIA	radioimmunoassay
ROI	region of interest
RTT	rat tail tendon
SDFT	superficial digital flexor tendon
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TB	thoroughbred
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of matrix metalloproteinase
TT	turkey tendon

APPENDIX TWO

Equine Bone, Cartilage and Ligament Cadaver Specimens

Bone and Cartilage Specimens

Horse	Age	Gender	Exercise Intensity Prior to Euthanasia	Cartilage Score			
				Right		Left	
				Cr	C3	Cr	C3
Racehorses:							
R1	7	Gelding	High	2	1	3	1
R2	7	Mare	High	0	1	0	0
R3	8	Gelding	High	1	2	1	3
R4	4	Colt	High	2	0	2	0
R5	6	Colt	High	0	1	0	1
R6	7	Gelding	High	1	1	0	1
R7	7	Colt	High	0	2	0	2
R8	7	Colt	High	1	2	1	2
R9	6	Colt	High	0	2	0	2
R10	6	Colt	High	0	2	0	2
R11	7	Gelding	High	0	2	0	1
R12	4	Gelding	High	0	2	0	1
R13	4	Colt	High	1	2	3	3
R14	4	Filly	High	0	0	0	0
Non-Racehorses:							
1	17	Mare	Light	0	0	0	0
2	NA	Gelding	NA	1	1	1	1
3	15	Gelding	Light	0	0	0	0
4	22	Gelding	Light	1	1	1	1
5	9	Mare	Medium	2	2	2	2
6	12	Gelding	Medium	0	0	0	0
7	10	Mare	NA	0	2	1	2
8	11	Mare	Light	0	0	0	0
9	8	Mare	NA	0	0	0	0
10	NA	Gelding	NA	0	0	0	0
11	3	Gelding	Light	0	0	0	0
12	6	Gelding	Medium	1	1	1	1
13	11	Mare	Light	1	2	2	2
14	17	Gelding	Medium	0	0	0	0
15	7	Gelding	NA	1	2	1	2
16	17	Gelding	Medium	0	1	0	1
17	14	Gelding	Light	0	0	0	3

Ligament Specimens

Horse	Age	Gender	Exercise Intensity Prior to Euthanasia	Ligament Score			
				Right		Left	
				DL	DM	DL	DM
Racehorses:							
R7	7	Colt	High	3	0	1	0
R8	7	Colt	High	2	0	3	0
R9	6	Colt	High	2	0	2	0
R10	6	Colt	High	2	0	2	0
R11	7	Gelding	High	0	0	0	0
R12	4	Gelding	High	2	0	1	0
R13	4	Colt	High	3	0	4	0
R14	4	Filly	High	0	0	0	0
R15	5	Gelding	High	1	0	0	0
R16	9	Gelding	High	2	0	0	0
R17	12	Gelding	High	2	0	1	0
R18	8	Gelding	High	3	1	3	0
R19	3	Gelding	High	1	0	2	0
R20	4	Filly	High	1	0	0	0
R21	4	Gelding	High	2	0	3	0
R22	3	Colt	High	1	0	2	0
R23	7	Gelding	High	0	0	0	0
R24	2	Colt	High	0	0	0	0
Non-Racehorses:							
2	NA	Gelding	NA	0	0	0	0
3	15	Gelding	Light	0	0	0	0
4	22	Gelding	Light	3	1	3	1
5	9	Mare	Medium	0	0	0	0
6	12	Gelding	Medium	0	0	0	0
7	10	Mare	NA	1	0	0	0
8	11	Mare	Light	1	0	4	0
9	8	Mare	NA	3	1	0	0
10	NA	Gelding	NA	0	0	0	0
11	3	Gelding	Light	4	0	0	0
12	6	Gelding	Medium	2	0	1	0
13	11	Mare	Light	0	0	0	0
14	17	Gelding	Medium	0	0	0	0
15	7	Gelding	NA	0	0	1	0
16	17	Gelding	Medium	1	0	0	2
17	14	Gelding	Light	1	0	2	0
18	9	Gelding	Medium	0	0	0	0
19	6	Gelding	NA	3	0	0	0
20	4	Gelding	Medium	0	0	0	0
21	13	Mare	Light	2	1	3	1
22	8	Gelding	Light	3	0	3	0
23	12	Gelding	Light	0	0	0	0
24	10	Gelding	Medium	0	0	0	0

Ligament and Cartilage Specimen Pathology Scores

Horse	Age	Gender	Ligament Score				Cartilage Score			
			Right		Left		Right		Left	
			DL	DM	DL	DM	Cr	C3	Cr	C3
Racehorses:										
R7	7	C	3	0	1	0	0	2	0	2
R8	7	C	2	0	3	0	1	2	1	2
R9	6	C	2	0	2	0	0	2	0	2
R10	6	C	2	0	2	0	0	2	0	2
R11	7	G	0	0	0	0	0	2	0	1
R12	4	G	2	0	1	0	0	2	0	1
R13	4	C	3	0	4	0	1	2	3	3
R14	4	F	0	0	0	0	0	0	0	0
R15	5	G	1	0	0	0	0	1	0	2
R16	9	G	2	0	0	0	1	4	1	4
R17	12	G	2	0	1	0	1	2	1	3
R18	8	G	3	1	3	0	1	0	1	4
R19	3	G	1	0	2	0	0	0	0	1
R20	4	F	1	0	0	0	1	1	0	0
R21	4	G	2	0	3	0	0	2	0	1
R22	3	C	1	0	2	0	0	0	0	0
R23	7	G	0	0	0	0	1	2	1	2
R24	2	C	0	0	0	0	0	0	0	1
Non-racehorses:										
2	NA	G	0	0	0	0	1	1	1	1
3	15	G	0	0	0	0	0	0	0	0
4	22	G	3	1	3	1	1	1	1	1
5	9	M	0	0	0	0	2	2	2	2
6	12	G	0	0	0	0	0	0	0	0
7	10	M	1	0	0	0	0	2	1	2
8	11	M	1	0	4	0	0	0	0	0
9	8	M	3	1	0	0	0	0	0	0
10	NA	G	0	0	0	0	0	0	0	0
11	3	G	4	0	0	0	0	0	0	0
12	6	G	2	0	1	0	1	1	1	1
13	11	M	0	0	0	0	1	2	2	2
14	17	G	0	0	0	0	0	0	0	0
15	7	G	0	0	1	0	1	2	1	2
16	17	G	1	0	0	2	0	1	0	1
17	14	G	1	0	2	0	0	0	0	3
18	9	G	0	0	0	0	1	2	1	2
19	6	G	3	0	0	0	0	0	0	0
20	4	G	0	0	0	0	0	0	0	0
21	13	M	2	1	3	1	0	1	1	1
22	8	G	3	0	3	0	0	0	0	0
23	12	G	0	0	0	0	0	0	1	3
24	10	G	0	0	0	0	0	1	0	1

APPENDIX THREE

Right and Left Differences – Bone and Ligament

BONE:

Radial Carpal Bone

Layer	Raced Right (Mean+/-SEM)	Raced Left (Mean+/-SEM)	P Value	Non-raced Right (Mean+/-SEM)	Non-raced Left (Mean+/-SEM)	P Value
Bone Mineral Density: Whole Bone (g/cm^3)						
a	0.92+/-0.01	0.92+/-0.01	0.88	0.91+/-0.02	0.95+/-0.02	0.05
b	0.82+/-0.02	0.78+/-0.02	0.52	0.77+/-0.03	0.84+/-0.03	0.12
c	0.72+/-0.02	0.77+/-0.05	0.21	0.68+/-0.03	0.72+/-0.04	**0.04**
d	0.64+/-0.02	0.67+/-0.02	0.33	0.60+/-0.03	0.63+/-0.03	**0.04**
Bone Mineral Density: Region of Interest (ROI) (g/cm^3)						
a	1.17+/-0.03	1.15+/-0.03	0.47	1.15+/-0.03	1.19+/-0.03	0.18
b	1.008+/-0.04	1.02+/-0.03	0.41	0.95+/-0.05	1.08+/-0.06	0.11
c	0.85+/-0.04	0.89+/-0.03	0.82	0.81+/-0.05	0.86+/-0.06	0.07
d	0.71+/-0.04	0.76+/-0.3	0.18	0.68+/-0.03	0.71+/-0.04	0.19
Total Calcium and Inorganic Phosphate (%)						
a	57.17+/-1.25	54.55+/-4.19	0.84	55.78+/-2.08	48.86+/-3.79	**0.03**
b	58.77+/-1.85	55.04+/-2.52	0.18	50.79+/-3.6	50.79+/-4.31	0.73
c	56.08+/-2.7	58.44+/-1.72	0.41	46.88+/-1.01	46.13+/-1.13	0.99
d	53.34+/-0.96	53.68+/-1.15	0.84	51.37+/-1.17	51.02+/-1.31	0.93
Cross-link: diHydroxy-lysinonorleucine (diHLNL) (cross-link mole/ mole collagen)						
a	0.12+/-0.008	0.11+/-0.01	0.24	0.16+/-0.03	0.14+/-0.03	0.22
b	0.63+/-0.24	0.63+/-0.28	0.55	0.40+/-0.08	0.29+/-0.06	0.16
c	0.36+/-0.12	0.53+/-0.22	0.22	0.22+/-0.04	0.25+/-0.07	0.47
d	0.32+/-0.07	0.35+/-0.11	0.72	0.13+/-0.02	0.2+/-0.05	0.19
Cross-link: Hydroxylysinonorleucine (HLNL) (cross-link mole/ mole collagen)						
a	0.07+/-0.01	0.07+/-0.01	0.74	0.11+/-0.02	0.12+/-0.02	0.77
b	0.29+/-0.09	0.29+/-0.11	0.96	0.36+/-0.06	0.28+/-0.05	0.17
c	0.16+/-0.03	0.23+/-0.07	0.25	0.21+/-0.04	0.25+/-0.06	0.33
d	0.19+/-0.03	0.22+/-0.05	0.45	0.14+/-0.03	0.20+/-0.04	0.30
Cross-link: Hydroxylysyl-Pyridinoline (HL-Pyr) (cross-link mole/ mole collagen)						
a	0.15+/-0.01	0.13+/-0.02	0.44	0.29+/-0.03	0.22+/-0.03	0.49
b	0.15+/-0.04	0.14+/-0.04	0.66	0.19+/-0.01	0.17+/-0.01	**0.04**
c	0.09+/-0.01	0.10+/-0.02	0.53	0.15+/-0.03	0.12+/-0.02	0.58
d	0.16+/-0.06	0.08+/-0.02	0.25	0.10+/-0.01	0.12+/-0.03	0.54

Layer	Raced Right	Raced Left	P Value	Non-raced Right	Non-raced Left	P Value
Cross-link: Lysyl- Pyridinoline (Lys-Pyr) (cross-link mole/ mole collagen)						
a	0.05+/-0.008	0.04+/-0.01	0.24	0.08+/-0.01	0.07+/-0.01	0.60
b	0.04+/-0.24	0.04+/-0.28	0.55	0.08+/-0.006	0.07+/-0.007	0.12
c	0.03+/-0.12	0.03+/-0.22	0.22	0.07+/-0.01	0.06+/-0.01	0.89
d	0.07+/-0.07	0.02+/-0.11	0.71	0.05+/-0.006	0.77+/-0.01	0.23
Hydroxylysine (Hylys mole/ mole collagen)						
a	12.69+/-2.63	10.11+/-1.92	0.12	7.71+/-0.77	6.40+/-1.09	0.056
b	12.11+/-1.08	13.09+/-2.33	0.36	10.23+/-1.64	9.62+/-2.16	0.99
c	13.89+/-1.34	15.4+/-2.23	0.68	10.36+/-1.72	8.86+/-1.58	0.058
d	12.24+/-1.5	14.14+/-2.17	0.09	8.27+/-1.24	7.44+/-0.74	0.78
Collagen Content (%)						
a	94.8+/-4.73	112.5+/-13.38	0.23	103.3+/-18.93	91.76+/-9.87	0.61
b	74.45+/-2.04	78.02+/-2.68	0.11	77.4+/-5.59	83.6+/-9.96	0.36
c	72.19+/-2.36	69.92+/-3.52	0.71	80.08+/-8.26	79.79+/-10.62	0.81
d	72.57+/-2.36	70.55+/-3.9	0.64	79.64+/-10.54	76.98+/-11.43	0.53
Type I Procollagen Propeptide (PICP) (µg/l)						
a	100+/-16.01	106.1+/-15.61	0.41	109.4+/-21.31	81.55+/-15.05	0.57
b	81.67+/-6.3	101.7+/-16.89	0.11	84.77+/-15.42	67+/-7.21	0.22
c	83.06+/-9.79	93.61+/-19.77	0.41	82.73+/-16.89	65.1+/-7.25	0.41
d	90+/-12.1	101.7+/-17.21	0.14	87.05+/-18.02	63.25+/-4.68	0.27
Alkaline Phosphatase (ALP) (mg dry weight)						
a	129.9+/-11.8	106.6+/-18.46	0.11	61.64+/-16.39	55.8+/-13.6	0.57
b	58.44+/-11.1	50.78+/-10.67	0.19	22.54+/-5.55	16.6+/-3.08	0.75
c	40.44+/-5.91	41.22+/-8.67	0.92	18.82+/-4.83	14.4+/-1.77	0.97
d	35.89+/-7.23	41.677.81	0.11	19.36+/-5.10	12.5+/-1.12	0.27
Matrix Metalloproteinase: Total MMP-2 (% std)						
a	19.53+/-5.06	18.04+/-4.69	0.66	16.2+/-6.21	8.70+/-3.63	0.19
b	8.46+/-2.41	5.98+/-3.63	0.06	3.45+/-1.66	0.00	0.06
c	9.03+/-2.91	11.65+/-3.71	0.25	2.85+/-2.01	2.48+/-1.54	0.71
d	14.71+/-4.49	13.29+/-3.45	0.41	9.30+/-5.06	3.88+/-2.05	0.28
Matrix Metalloproteinase: Total MMP-9 (% std)						
a	79.05+/-15.03	90.64+/-11.57	0.34	66.48+/-16.99	55.44+/-13.52	0.37
b	71.57+/-6.97	91.15+/-14.45	0.06	43.69+/-15.44	42.94+/-15.5	0.68
c	67.46+/-7.72	72.13+/-9.25	0.40	58.13+/-22.51	28.6+/-9.74	0.32
d	72.45+/-4.86	85.26+/-11.79	0.18	34.27+/-12.21	25.95+/-7.35	0.81
Tissue Inhibitors of Matrix Metalloproteinases: TIMP-2 (% std)						
a	15.41+/-5.12	19.39+/-6.47	0.18	26.3+/-27.34	27.34+/-5.11	0.78
b	12.02+/-2.86	13.16+/-3.277	0.97	25.03+/-8.18	22.75+/-8.14	0.28
c	17.86+/-5.48	17.63+/-8.253	0.98	10.77+/-2.64	14.2+/-2.42	0.09
d	18.77+/-7.09	16.71+/-6.24	0.19	21.34+/-5.87	16.67+/-4.83	**0.04**

Layer	Raced Right	Raced Left	P Value	Non-raced Right	Non-raced Left	P Value
DSC: Whole Peak – Enthalpy (J/ mg dry weight)						
a	67.31+/-2.2	63.03+/-5.03	0.41	69.87+/-6.19	64.55+/-7.43	0.89
b	53.37+/-5.39	66.08+/-3.31	**0.008**	82.26+/-8.09	58.73+/-5.28	0.08
c	82.1+/-6.96	55.46+/-3.49	**0.03**	74.3+/-8.03	63.24+/-5.51	0.45
d	69.83+/-4.76	68.59+/-9.1	0.91	82.67+/-12.4	73.38+/-7.66	0.38
DSC: Whole Peak – Tmax (^{o}C)						
a	59.74+/-1.5	60.01+/-0.79	0.89	58.38+/-0.92	60.78+/-1.99	0.57
b	61.05+/-2.57	57.92+/-0.94	0.14	57.23+/-1.42	57.77+/-0.8	0.86
c	57.62+/-0.88	57.51+/-0.66	0.78	57.97+/-0.82	58.89+/-1.77	0.63
d	57.78+/-0.79	57.17+/-1.19	0.62	58.47+/-1.6	59.82+/-2.2	0.48

3rd Carpal Bone

Layer	Raced Right (Mean+/-SEM)	Raced Left (Mean+/-SEM)	P Value	Non-raced Right (Mean+/-SEM)	Non-raced Left (Mean+/-SEM)	P Value
Bone Mineral Density: Whole Bone (g/cm^3)						
a	0.93+/-0.01	0.94+/-0.008	**0.04**	0.90+/-0.02	0.91+/-0.02	0.73
b	0.82+/-0.02	0.83+/-0.03	0.38	0.79+/-0.03	0.81+/-0.03	0.12
c	0.70+/-0.02	0.69+/-0.03	0.62	0.59+/-0.02	0.60+/-0.02	0.73
d	0.69+/-0.02	0.66+/-0.02	0.61	0.59+/-0.02	0.60+/-0.01	0.79
Bone Mineral Density: Region of Interest (ROI) (g/cm^3)						
a	1.10+/-0.04	1.14+/-0.05	0.16	1.03+/-0.02	0.99+/-0.03	0.14
b	1.05+/-0.36	1.06+/-0.03	0.39	1.01+/-0.05	1.04+/-0.04	0.37
c	0.88+/-0.03	0.88+/-0.05	0.89	0.73+/-0.04	0.73+/-0.04	0.88
d	0.85+/-0.03	0.83+/-0.03	0.85	0.71+/-0.03	0.75+/-0.02	0.38
Total Calcium and Inorganic Phosphate (%)						
a	56.09+/-0.80	53.78+/-1.66	0.28	55.76+/-2.49	55.23+/-0.94	0.83
b	54.71+/-2.44	56.45+/-0.7	0.51	51.05+/-3.04	54.55+/-1.53	0.32
c	52.98+/-0.51	54.07+/-0.94	0.11	45.58+/-0.83	45.2+/-0.86	**0.005**
d	54.55+/-0.83	54.07+/-1.52	0.77	49.8+/-1.33	48.83+/-3.94	0.71
Cross-link: diHydroxy-lysinonorleucine (diHLNL) (cross-link mole/ mole collagen)						
a	0.13+/-0.02	0.11+/-0.01	0.45	0.15+/-0.05	0.15+/-0.03	0.75
b	0.54+/-0.20	0.48+/-0.20	0.31	0.36+/-0.06	0.33+/-0.07	0.54
c	0.45+/-0.14	0.53+/-0.18	0.96	0.25+/-0.08	0.23+/-0.05	0.76
d	0.35+/-0.38	0.38+/-0.09	0.62	0.17+/-0.05	0.21+/-0.07	0.28
Cross-link: Hydroxylysinonorleucine (HLNL) (cross-link mole/ mole collagen)						
a	0.07+/-0.03	0.06+/-0.02	0.54	0.16+/-0.19	0.15+/-0.09	0.68
b	0.26+/-0.08	0.29+/-0.09	0.055	0.35+/-0.05	0.34+/-0.05	0.89
c	0.22+/-0.05	0.25+/-0.07	0.84	0.25+/-0.08	0.24+/-0.04	0.83
d	0.21+/-0.04	0.23+/-0.06	0.59	0.18+/-0.06	0.24+/-0.08	0.65

Layer	Raced Right	Raced Left	P Value	Non-raced Right	Non-raced Left	P Value
Cross-link: Hydroxylysyl-Pyridinoline (HL-Pyr) (cross-link mole/ mole collagen)						
a	0.13+/-0.02	0.11+/-0.01	0.31	0.17+/-0.03	0.17+/-0.01	0.76
b	0.13+/-0.04	0.13+/-0.04	0.76	0.21+/-0.04	0.17+/-0.01	0.24
c	0.09+/-0.02	0.11+/-0.03	0.53	0.16+/-0.04	0.12+/-0.01	0.23
d	0.08+/-0.01	0.09+/-0.01	0.31	0.12+/-0.02	0.14+/-0.03	0.95
Cross-link: Lysyl-Pyridinoline (Lys-Pyr) (cross-link mole/ mole collagen)						
a	0.03+/-0.006	0.03+/-0.004	0.9	0.06+/-0.01	0.06+/-0.006	0.07
b	0.03+/-0.006	0.04+/-0.007	0.54	0.09+/-0.01	0.08+/-0.007	0.09
c	0.02+/-0.003	0.02+/-0.005	0.67	0.08+/-0.01	0.06+/-0.006	0.08
d	0.02+/-0.003	0.03+/-0.003	0.53	0.06+/-0.01	0.07+/-0.015	0.07
Hydroxylysine (Hylys mole/ mole collagen)						
a	11.67+/-2.59	10.33+/-1.7	0.41	6.99+/-1.12	6.50+/-0.65	0.79
b	14.99+/-2.42	12.2+/-1.61	0.50	10.71+/-2.17	9.67+/-1.75	0.40
c	10.91+/-1.17	13.59+/-1.60	0.09	8.49+/-1.58	7.64+/-1.8	0.67
d	13.84+/-1.04	12.27+/-1.00	0.11	7.891+/-1.04	6.68+/-1.00	0.45
Collagen Content (%)						
a	94.67+/-5.85	96.17+/-4.35	0.86	89.72+/-8.22	96.3+/-4.91	0.37
b	77.11+/-3.13	78.49+/-3.18	0.96	79.64+/-9.97	79.63+/-5.66	0.76
c	69.84+/-2.13	70.84+/-3.68	0.61	84.76+/-11.95	85.69+/-8.83	0.94
d	96.44+/-1.6	70.2+/-2.41	0.71	71.66+/-11.28	77.69+/-11.4	0.86
Type I Procollagen Propeptide (PICP) (µg/l)						
a	118.1+/-15.01	135.6+/-21.61	0.22	103.2+/-20.32	87.55+/-19.99	0.69
b	107.5+/-16.6	125.8+/-19.85	0.22	78.64+/-12.76	72.75+/-11.73	0.68
c	91.94+/-18.52	97.78+/-14.75	0.30	71.36+/-11.27	61.75+/-4.74	0.44
d	100.8+/-17.37	100.6+/-15.69	0.94	67.73+/-5.95	66.5+/-5.24	0.85
Alkaline Phosphatase (ALP) (mg dry weight)						
a	134.7+/-25.57	159.8+/-14.83	0.37	40.82+/-9.61	36.2+/-11.27	0.80
b	61.57+/-10.17	74.56+/-15.49	0.31	17.45+/-3.23	17.5+/-3.931	0.53
c	48.78+/-6.13	55.33+/-10.53	0.50	16.27+/-2.70	14.3+/-2.28	0.79
d	43.44+/-8.51	64.44+/-10.94	0.10	15.82+/-2.72	14.8+/-2.36	0.81
Matrix Metalloproteinase: Total MMP-2 (% std)						
a	32.32+/-8.46	20.6+/-7.34	**0.01**	15.53+/-5.41	0.81+/-0.47	**0.03**
b	11.75+/-5.47	22.23+/-6.52	0.07	1.42+/-1.04	1.72+/-0.94	0.72
c	20.44+/-5.78	21.24+/-7.82	0.83	9.23+/-5.76	7.65+/-4.09	0.85
d	21.61+/-6.46	26.69+/-8.33	0.15	5.7+/-4.15	10.3+/-6.69	0.14
Matrix Metalloproteinase: Total MMP-9 (% std)						
a	127.2+/-10.84	138.8+/-20.79	0.57	67.31+/-16.37	67.21+/-19.54	0.70
b	89+/-7.08	88.75+/-13.35	0.98	47.93+/-13.24	31.35+/-10.65	0.13
c	101+/-6.88	96.95+/-8.34	0.64	57.38+/-19.97	37.04+/-14.91	0.23
d	103.6+/-18.94	151.1+/-31.62	0.07	33.2+/-9.161	70.63+/-30.92	0.14

Layer	Raced Right	Raced Left	P Value	Non-raced Right	Non-raced Left	P Value
Tissue Inhibitors of Matrix Metalloproteinases: TIMP-2 (% std)						
a	18.56+/-5.22	17.97+/-4	0.75	17.12+/-3.6	23.98+/-9.23	0.69
b	13.72+/-5.26	16.87+/-4.57	0.75	11.48+/-2.8	13.18+/-3.78	0.37
c	14.17+/-3.4	11.4+/-4.28	0.254	11.94+/-1.5	17.05+/-6.65	0.49
d	14.04+/-5.99	14.68+/-4.98	0.64	11.91+/-3.77	15.48+/-3.87	0.22
DSC: Whole Peak – Enthalpy (J/ mg dry weight)						
a	60.92+/-4.39	70.07+/-4.49	0.24	62.78+/-6.94	70.87+/-8.51	0.26
b	58.59+/-4.22	59.74+/-5.03	0.89	93.31+/-16.7	78.39+/-12.71	0.49
c	71.14+/-9.43	71.25+/-3.38	0.99	70.26+/-6.24	72.82+/-2.27	0.87
d	63.51+/-5.54	63.66+/-2.81	0.98	74.18+/-2.88	69.43+/-3.68	0.34
DSC: Whole Peak – Tmax (^{o}C)						
a	59.49+/-1.18	58.63+/-0.71	0.27	60.41+/-1.52	59.05+/-0.88	0.20
b	59.09+/-1.42	58.32+/-1.46	0.77	61.15+/-2.83	58.36+/-0.85	0.37
c	57.35+/-0.78	56.92+/-0.62	0.30	59.66+/-1.19	57.71+/-1.08	0.22
d	59.83+/-1.91	56.57+/-1.33	0.17	58.57+/-1.33	57.39+/-1.36	0.07

LIGAMENT:

Layer	Raced Right (Mean+/-SEM)	Raced Left (Mean+/-SEM)	P Value	Non-raced Right (Mean+/-SEM)	Non-raced Left (Mean+/-SEM)	P Value
Cross-link: Total Immature (diHLNL & HLNL) (cross-link mole/ mole collagen)						
DL	0.05+/-0.02	0.03+/-0.009	0.49	0.0	0.01+/-0.007	Too Few
DM	0.03+/-0.008	0.04+/-0.01	0.65	0.008+/-0.002	0.006+/-0.002	0.34
Cross-link: Total Mature (HL-Pyr & Lys-Pyr) (cross-link mole/ mole collagen)						
DL	0.47+/-0.10	0.65+/-0.13	0.14	0.57+/-0.23	0.61+/-0.24	0.06
DM	1.14+/-0.43	0.68+/-0.21	0.37	0.67+/-0.16	0.96+/-0.29	0.42
Cross-link: HHL (cross-link mole/ mole collagen)						
DL	0.07+/-0.01	0.05+/-0.01	0.24	0.05+/-0.02	0.08+/-0.02	0.99
DM	0.05+/-0.009	0.09+/-0.03	0.11	0.04+/-0.009	0.02+/-0.007	0.66
Collagen Content (%)						
DL	73.42+/-2.89	74.17+/-2.89	0.95	79.16+/-15.2	66.87+/-3.80	0.24
DM	66.94+/-4.71	71.75+/-2.531	0.5	77.13+/-6.38	81.98+/-9.35	0.69
Type I Procollagen Propeptide (PICP) (µg/l)						
DL	114+/-37.12	130.7+/-30.05	0.62	87.5+/-62.5	222.2+/-71.63	Too Few
DM	11.8+/-27.92	88.5+/-37.44	0.36	196.1+/-75.82	153.9+/-82.03	0.73
Matrix Metalloproteinase: Total MMP-2 (% std)						
DL	65.16+/-17.06	49.5+/-10.53	0.53	91.64+/-23.34	48.56+/-8.948	0.62
DM	43.51+/-8.13	39.2+/-8.27	0.52	69.97+/-10.25	36.25+/-8.86	0.10

Layer	Raced Right	Raced Left	P Value	Non-raced Right	Non-raced Left	P Value
Matrix Metalloproteinase: Total MMP-9 (% std)						
DL	31.6+/-9.82	27.56+/-3.83	0.92	21.45+/-3.58	25.76+/-3.27	0.88
DM	17.39+/-4.91	28.69+/-8.12	0.16	24.81+/-3.97	38.8+/-9.04	0.08
Tissue Inhibitors of Matrix Metalloproteinases: TIMP-2 (% std)						
DL	19.15+/-0.0	36.66+/-9.79	Too Few	28.67+/-3.23	32.72+/-3.87	0.59
DM	31.57+/-5.24	36.96+/-7.80	0.37	25.89+/-4.65	26.34+/-7.13	0.23
DSC: Enthalpy (J/ mg dry weight)						
DL	51.09+/-7.62	50.86+/-10.56	0.69	56.43+/-5.9	56.16+/-9.44	0.69
DM	49.42+/-4.88	50.66+/-7.06	0.80	69.7+/-11.11	80.35+/-9.34	0.47
DSC: Tmax (^{o}C)						
DL	70.38+/-0.18	70.5+/-0.348	0.77	69.44+/-0.20	69.67+/-0.11	0.20
DM	70.13+/-0.18	69.89+/-0.16	0.40	69.33+/-0.35	69.41+/-0.34	0.80

APPENDIX FOUR

Materials

All reagents were analar grade or equivalent.

Agar Scientific UK Ltd.

Araldite CY212, osmium tetroxide, propylene oxide, paraformaldehyde, sodium cacodylate, uranyl acetate.

Aldrich Chemical Company Ltd.

Coomassie Brilliant Blue, toludine blue.

Ambersil UK Ltd.

Curing agent A, silcoset 105.

Amersham International Plc.

Sodium boro(3^{H})hydride.

BDH UK Ltd.

Ammonium persulphate, chloramine-T, citric acid, glycerol, pyridine, sodium acetate trihydrate, sodium dodecyl sulphate, sodium periodate, sodium tetraborate, TEMED, thiourea, triethanolamine, trisodium citrate, urea.

Bio-rad, UK.

Acrylamide, tricine sample buffer.

Calbiochem UK Ltd.

Recombinant MMP-2, and TIMP-2.

Fisons Ltd.

Acetic acid, ammonium hydrogen carbonate, butan-1-ol, ethanol, formic acid, glycine, hydrochloric acid, 2-merceptoethanol, methanol, perchloric acid, propan-2-ol, potassium dihydrogen orthophosphate, sodium chloride, sodium dihydrogen orthophosphate,

sodium hydrogen carbonate, sodium hydroxide, disodium hydrogen orthophosphate, sulphuric acid.

Giltspur Scientific Ltd.

Benzoyl peroxide, dimethyl toluidin, methyl methacrylate, polymethyl methacrylate.

Pharmacia LKB Biochrom Ltd.

Buffers and reagents for Alpha Plus amino acid analyser.

Sigma Ltd.

Alanine, APMA, bromophenol blue, calcium chloride, chondroitin-4-sulphate, dermatan sulphate, 1,9-dimethylmethylene blue, 4-dimethylaminobenzaldehyde, EDTA, gelatin, guanidine hydrochloride, glucosamine, hydroxyproline, lysine, ninhydrin, papain, potassium borohydride, proline, Trizma-base, Tris-HCL, tritonX-100.

APPENDIX FIVE

Solutions

All solutions used deionised water (Milli RO6/ Milli Q50 purification system, Millipore) unless otherwise stated.

Phosphate Buffered Saline (PBS)

NaCL	8g
KCl	0.2g
KH_2PO_4	0.2g
Na_2HPO_4	1.15g

to 1 litre, pH7.4

Gelatin and Reverse Zymography

All reagents were from Sigma (Poole, U.K.) except for acrylamide/bis, which was from Biorad (Herts, U.K.).

Non-reducing Sample Buffer

125mM Tris-HCL pH 6.8

15% glycerol (v/v)

0.01% SDS (w/v)

1% Bromophenol blue (w/v)

MMP Proteolysis Buffer

50mM Tris-HCL pH 7.8

50mM $CaCl_2$

500mM NaCl

Coomassie Blue Stain

1% Coomassie blue (w/v)

40% methanol (v/v)

10% acetic acid (v/v)

Destain

7.5% acetic acid

10% methanol

Laemlii Running Buffer

25mM Tris-HCL pH 8.3

0.5% SDS (w/v)

192mM glycine

Gelatin Zymography - Resolving Gel (Quantity for 2 gels)

dH_2O	5ml
1.5M Tris-HCL pH 8.8	3.6ml
29:1 acrylamide/bis	4.8ml
10% SDS	100µl
7.5mg/ml gelatin solution	1.5ml
10% ammonium persulfate (APS)	75µl
Tetramethylethylendiamine (TEMED)	10µl

Heat the gelatin solution to 60°C until the gelatin has dissolved. Just prior to casting the gels add the warm gelatin solution, TEMED and APS to the rest of the solution and mix thoroughly.

Gelatin Zymography - Stacking gel (Quantity for 4 gels)

dH_2O	5.9ml
0.5 M Tris-HCL pH 6.8	2.6ml
29:1 acrylamide/bis	3ml
10% SDS	100µl
10% APS	75µl
TEMED	15µl

As described previously add the TEMED and APS just prior to casting the gels and mix thoroughly.

Reverse Zymography - Recombinant MMP-2 Resolving Gel (Quantity for 1 gel)

dH_2O	0.8ml
1.5 M Tris-HCL pH 8.8	1.8ml
29:1 acrylamide/bis	3.6ml
10% SDS	50µl
7.5mg/ml gelatin solution	1.25ml
MMP-2 (0.1µg/µl)	5µl
10% APS	35µl
TEMED	5µl

As previously described warm gelatin solution, TEMED and APS are added to the recombinant MMP-2 resolving gel mixture prior to casting.

Reverse Zymography – 10% SDS-PAGE Gel (Quantity for 1 gel)

dH_2O	2.05ml
1.5 M Tris-HCL pH 8.8	2.6ml
29:1 acrylamide/bis	3.6ml
10% SDS	50µl

Reverse Zymography - Stacking gel (Quantity for 4 gels)

dH_2O	5ml
1.5 M Tris-HCL pH 6.8	2.6ml
29:1 acrylamide/bis	3.9ml
10% SDS	200µl
10% APS	150µl
TEMED	30µl

As described previously add the TEMED and APS just prior to casting the gels and mix thoroughly.

Hydroxyproline Analysis

Diluent

Propan-2-ol	2 volumes
dH_2O	1 volume

Stock Buffer

Sodium acetate trihydrate	57g
Trisodium citrate	37.5g
Citric acid	5.5g
Propan-2-ol	400ml

Dissolve solids in approximately 500mls dH_2O, add propan-2-ol and make up to 1l with dH_2O.

Oxidant

Chloramine-T	2.8g
dH_2O	40ml
Stock buffer	200ml

Dissolve chloramine-T in dH_2O then dilute with stock buffer. Prepare fresh daily.

Colour Reagent

4-dimethylaminobenzaldehyde	30g
Perchloric acid	45ml
Propan-1-ol	250ml

Dissolve solids in perchloric acid then add propan-2-ol. Stable at 4°C for up to 5 days in the dark.

Stock Standard

L-hydroxyproline	500mgs
0.01M Hydrochloric acid	500mls

Stored at -20°C in 5ml aliquots until required.

APPENDIX SIX

Glossary of Mechanical Terms

(Adapted from Woo *et al*, 1990)

Creep: A viscoelastic behaviour characterised by a change in strain or deformation of a specimen with time under a constant applied stress.

Deformation (millimetre - mm): The change in dimensions of specimen under external tensile loading. (For example; the increase in length of a bone-ligament-bone complex under tensile loading).

Energy Absorbed At Failure (newton.metre – Nm=J): The total energy absorbed by the specimen under tensile loading from zero loading until failure. A structural property, represented by the area under the load-deformation curve up to the point of failure.

Hysteresis: The area on a load-deformation curve between the tension and relaxation curves. It represents the internal energy loss (mainly converted to heat energy) between the loading and unloading phases.

Linear Stiffness (newton per millimetre – N/mm): The slope of the load-deformation curve where the curve is most linear. A structural property representing the tensile load required to elongate the specimens by a unit length.

Load (newton – N): External force applied to a specimen.

Material Properties: Tensile properties of the ligament substance, as a material, represented by the stress-strain curve.

Newton: 1 newton produces an acceleration of $1ms^{-2}$ in a mass of 1kg.

Pascal: 1 pascal= a pressure acting uniformly on an area, which exerts a force of one newton vertically on one metre^2 of the area.

Strain:

$$\text{Strain} = \frac{\text{change in specimen dimension under external loading}}{\text{initial dimensions}}$$

Strain rate (percent per second - %/s): The rate at which a specimen is deformed.

Stress (Newton per square millimetre – N/mm^2=1MPa):

$$\text{Stress} = \frac{\text{load in a specimen}}{\text{cross-sectional area perpendicular to axis of loading}}$$

Structural (Mechanical)Properties: The tensile properties of the bone-ligament-bone complex as a structural unit. Represented by the load-deformation curve.

Tangent Modulus (megapascal-MPa): The slope of a line tangent to a segment of the stress-strain curve within a specified range of strain.

Tensile Properties: Describe the behaviour of a material under tensile load.

Tensile Strength (newton per square millimetre – Mpa):

$$\text{Tensile Strength} = \frac{\text{load at failure}}{\text{original cross-sectional area perpendicular to axis of loading}}$$

Ultimate Load (newton – N): The maximum load that can be sustained by a specimen prior to failure.

Ultimate Deformation (millimetre – mm): The maximum deformation that can be sustained by a specimen prior to failure.

Ultimate Strain (percent - %):

$$\text{Ultimate Strain} = \frac{\text{ultimate deformation}}{\text{initial length of specimen}}$$

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